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## Description

Field of the invention

The present invention relates to the field of recombinant DNA technology, to means and methods utilizing such technology in the discovery of the DNA sequence and deduced amino acid sequence for human immune interferon and to its production and to the various products of such production and their uses.

More particularly, the present invention relates to the isolation and identification of DNA sequences encoding human immune interferon and to the construction of recombinant DNA expression vehicles containing such DNA sequences operably linked to expression-effecting promoter sequences and to the expression vehicles so constructed. In another aspect, the present invention relates to host culture systems, such as various microorganism and vertebrate cell cultures transformed with such expression vehicles and thus directed in the expression of the DNA sequences referred to above. In yet other aspects, this invention relates to the means and methods of converting the end products of such expression to novel entities, such as pharmaceutical compositions, useful for the prophylactic or therapeutic treatment of humans. In preferred embodiments, this invention provides particular expression vehicles that are sequenced properly such that human immune interferon is produced and secreted from the host cell in mature form. In addition, this invention relates to various processes useful for producing said DNA sequences, expression vehicle, host culture systems and end products and entities thereof and to specific and associated embodiments thereof.

The present invention arises in part from the discovery of the DNA sequence and deduced amino acid sequence encoding human immune interferon. In addition, the present invention provides sequence information on the 3'- and 5'-flanking sequences of the human immune interferon gene, faciliating the *in vitro* linkage thereof into expression vehicles. In particular, there is provided the 5'-DNA segment encoding the putative endogenous signal polypeptide which immediately precedes the amino acid sequence of the putative mature human immune interferon. These discoveries, in turn, have enabled the development of the means and methods for producing, via recombinant DNA technology, sufficient amounts of human immune interferon, so as to enable, in turn, the determination of its biochemical properties and bioactivity.

The publications and other materials hereof used to illuminate the background of the invention, and in particular cases, to provide additional details respecting its practice are incorporated herein by reference, and for convenience, are numerically referenced by the following text and respectively grouped in the appended bibliography.

Background of the invention

A. Human immune interferon

Human interferons can be classified in three groups on the basis of different antigenicity and biological and biochemical properties.

The first group comprises a family of leukocyte interferons (α-interferon, LeIF or IFN-α), which are normally produced mainly by constituent cells of human blood upon viral induction. These have been microbially produced and found to be biologically active (1, 2, 3). Their biological properties have prompted their use in the clinic as therapeutic agents for the treatment of viral infections and malignant conditions (4).

In the second group is human fibroblast interferon (β-interferon, FIF or IFN-β), normally produced by fibroblasts upon viral induction, which has likewise been microbially produced and found to exhibit a wide range of biological activities (5). Clinical trials also indicate its potential therapeutic value. The leukocyte and fibroblast interferons exhibit very clear similarities in their biological properties despite the fact that the degree of homology at the amino acid level is relatively low. In addition, both groups of interferons contain from 165 to 166 amino acids and are acid stable proteins.

The human immune interferon (IFN-γ), otherwise also referred to as human gamma interferon, to which this invention is directed, is, in contrast to the α- and ≡-interferons, pH 2 labile, is produced mainly upon mitogenic induction of lymphocytes and is also clearly antigenically distinct. Until recently human immune interferon could only be detected in very minor levels, which evidently hampered its characterization. Recently, a rather extensive but still partial purification of human immune interferon has been reported (6). The compound was said to be produced from lymphocyte cultures stimulated with a combination of phytohaemagglutin and a phorbol ester and purified by sequential chromatographic separations. This procedure resulted in a product having a molecualr weight of 58,000.

Human immune interferon has been produced in very low amounts by translating mRNA in oocytes, showing interferon activity characteristic of human immune interferon and expressing the hope that immune interferon cDNA could be synthesized and cloned (7).

The amount of immune interferon obtained until now is certainly insufficient to carry out umabiguous experiments on the characterization and biological properties of the purified component. However, *in vitro* studies performed with crude preparations, as well as *in vivo* experiments with murine γ-interferon preparations, suggest that the primary function of immune interferon may be as an immunoregulatory agent (8, 9). Immune interferon has not only an antiviral and anticellular activity in common to all human interferons, but shows a potentiating effect on these activities with α- and β-interferon (10). Also, the *in*

*vitro* antiproliferative effect of γ-interferon on tumor cells is reported to be approximately 10- to 100-fold that of the other interferon classes (8, 11, 12). This result, together with its pronounced immunoregulatory role (8, 9), suggests a much more pronounced antitumoral potency for IFN-γ than for IFN-α and IFN-α. Indeed, *in vivo* experiments with mice and murine IFN-γ preparations show a clear superiority over antivirally induced interferons in its antitumoral effect against osteogenic sarcoma (13).

All of these studies, until the present invention, had to be performed with rather crude preparations, due to the very low availability. However, they certainly suggest very important biological functions for immune interferon. Not only has immune interferon a potent associated antiviral activity, but probably also a strong immunoregulatory and antitumoral activity, clearly pointing to a potentially very promising clinical candidate.

It was perceived that the application of recombinant DNA technology would be a most effective way of providing the requisite larger quantities of human immune interferon. Whether or not the materials so produced would include glycosylation which is considered characteristic of native, human derived material, they would probably exhibit bioactivity admitting of their use clinically in the treatment of a wide range of viral, neoplastic, and immunosuppressed conditions or diseases.

B. Recombinant DNA technology

Recombinant DNA technology has reached the age of some sophistication. Molecular biologists are able to recombine various DNA sequences with some facility, creating new DNA entities capable of producing copious amounts of exogenous protein product in transformed microbes. The general means and methods are in hand for the *in vitro* ligation of various blunt ended or "sticky" ended fragments of DNA, producing potent expression vehicles useful in transforming particular organisms, thus directing their efficient synthesis of desired exogenous product. However, on an individual product basis, the pathway remains somewhat tortuous and the science has not advanced to a stage where regular predictions of success can be made. Indeed, those who portend successful results without the underlying experimental basis, do so with considerable risk of inoperability.

The plasmid, a nonchromosomal loop of double-stranded DNA found in bacteria and other microbes, oftentimes in multiple copies per cell, remains a basic element of recombinant DNA technology. Included in the information encoded in the plasmid DNA is that required to reproduce the plasmid in daughter cells (i.e., an origin of replication) and ordinarily, one or more phenotypic selection characteristics such as, in the case of bacteria, resistance to antibiotics, which permit clones of the host cell containing the plasmid of interest to be recognized and preferentially grown in selective media. The utility of plasmids lies in the fact that they can be specifically cleaved by one or another restriction endonuclease or "restriction enzyme", each of which recognizes a different site on the plasmid DNA. Thereafter heterologous genes or gene fragments may be inserted into the plasmid by endwise joining at the cleavage site or at reconstructed ends adjacent to the cleavage site. Thus formed are so-called replicable expression vehicles. DNA recombination is performed outside the cell, but the resulting "recombinant" replicable expression vehicle, or plasmid, can be introduced into cells by a process known as transformation and large quantities of the recombinant vehicle obtained by growing the transformant. Moreover, where the gene is properly inserted with reference to portions of the plasmid which govern the transcription and translation of the encoded DNA message, the resulting expression vehicle can be used to actually produce the polypeptide sequence for which the inserted gene codes, a process referred to as expression.

Expression is initiated in a region known as the promoter which is recognized by and bound by RNA polymerase. In the transcription phase of expression, the DNA unwinds, exposing it as a template for initiated synthesis of messenger RNA from the DNA sequence. The messenger RNA is, in turn, translated into a polypeptide having the amino acid sequence encoded by the mRNA. Each amino acid is encoded by a nucleotide triplet or "codon" which collectively make up the "structural gene", i.e. that part which encodes the amino acid sequence of the expressed polypeptide product. Translation is initiated at a "start" signal (ordinarily ATG, which in the resulting messenger RNA becomes AUG). So-called stop codons define the end of translation and, hence, of production of further amino acid units. The resulting product may be obtained by lysing, if necessary, the host cell, in microbial systems, and recovering the product by appropriate purification from other proteins.

In practice, the use of recombinant DNA technology can express entirely heterologous polypeptides—so-called direct expression—or alternatively may express a heterologous polypeptide fused to a portion of the amino acid sequence of a homologous polypeptide. In the latter cases, the intended bioactive product is sometimes rendered bioinactive within the fused, homologous/heterologous polypeptide until it is cleaved in an extracellular environment. See British Patent Publ. No. 2007676A and Wetzel, *American Scientist 68*, 664 (1980).

C. Cell culture technology

The art of cell or tissue cultures for studying genetics and cell physiology is well established. Means and methods are in hand for maintaining permanent cell lines, prepared by successive serial transfers from isolate normal cells. For use in research, such cell lines are maintained on a solid support in liquid medium, or by growth in suspension containing support nutriments. Scale-up for large preparations seems to pose only mechanical problems. For further background, attention is directed to *Microbiology*, 2nd Edition,

Harper and Row, Publishers, Inc., Hagerstown, Maryland (1973) especially pp. 1122 et seq. and *Scientific American 245,* 66 *et seq.* (1981), each of which is incorporated herein by this reference.

Summary of the invention

The present invention is based upon the discovery that recombinant DNA technology can be used to successfully produce human immune interferon, preferably in direct form, and in amounts sufficient to initiate and conduct animal and clinical testing as prerequisites to market approval. The product is suitable for use, in all of its forms, in the prophylactic or therapeutic treatment of human beings for viral infections and malignant and immunosuppressed or immunodeficient conditions. Its forms include various possible oligomeric forms which may include associated glycosylation. The product is produced by genetically engineered transformant microorganisms or transformant cell culture systems. As used herein, the term "transformant microorganism" or "transformant cell" refers to a host into which has been introduced DNA, said DNA arising from exogenous DNA recombination and to the progeny of any such host which retains the DNA so introduced. Thus, the potential now exists to prepare and isolate human immune interferon in a more efficient manner than has been possible. One significant factor of the present invention, in its most preferred embodiments, is the accomplishment of genetically directing a microorganism or cell culture to produce human immune interferon to isolatable amounts, secreted from the host cell in mature form.

The present invention comprises the human immune interferon thus produced and the means and methods of its production. The present invention is further directed to replicable DNA expression vehicles harboring gene sequences encoding human immune interferon in expressible form. Further, the present invention is directed to microorganism strains or cell cultures transformed with the expression vehicles described above and to microbial or cell cultures of such transformed strains or cultures, capable of producing human immune interferon. In still further aspects, the present invention is directed to various processes useful for preparing said immune interferon gene sequences, DNA expression vehicles; microorganism strains and cell cultures and to specific embodiments thereof. Still further, this invention is directed to the preparation of fermentation cultures of said microorganisms and cell cultures. In addition, this invention is directed to the preparation of human immune interferon, as a direct expression product, secreted from the host cell in mature form. This approach may utilize the gene encoding the sequence of the mature human immune interferon plus the 5' flanking DNA encoding the signal polypeptide. The signal polypeptide is believed to aid in the transport of the molecule to the cellular wall of the host organisms where it is cleaved during the secretion process of the mature human interferon product. This embodiment enables the isolation and purification of the intended mature immune interferon without resort to involved procedures designed to eliminate contaminants of intracellular host protein or cellular debris.

Reference herein to the expression "mature human immune interferon" connotes the microbial or cell culture production of human immune interferon unaccompanied by the signal peptide or presequence peptide that immediately attends translation of the human immune interferon mRNA. A first recombinant human immune interferon, according to the present invention, is thus provided, having methionine as its first amino acid (present by virtue of the ATG start signal codon insertion in front of the structural gene) or, where the methionine is intra- or extracellularly cleaved, having its normally first amino acid cysteine. Mature human immune interferon can also be produced, in accordance herewith, together with a conjugated protein other than the conventional signal polypeptide, the conjugate being specifically cleavable in an intra- or extracellular environment. See British Patent Publication No. 2007676A. Finally, the mature human immune interferon can be produced by direct expression without the necessity of cleaving away any extraneous, superfluous polypeptide. This is particularly important where a given host may not, or not efficiently, remove a signal peptide where the expression vehicle is designed to express the mature human interferon together with its signal peptide. The thus produced mature human immune interferon is recovered and purified to a level fitting it for use in the treatment of viral, malignant, and immunosuppressed or immunodeficient conditions.

Human immune interferon was obtained according to the following:

1. Human tissues, for example human spleen tissue or peripheral blood lymphocytes, were cultured with mitogens to stimulate the production of immune interferon.

2. Cell pellets from such cell cultures were extracted in the presence of ribonuclease inhibitor to isolate all cytoplasmic RNA.

3. An oligo-dT column isolated the total messenger RNA (mRNA) in polyadenylated form. This mRNA was size-fractionated using sucrose density gradient and acid-urea gel electrophoresis.

4. The appropriate mRNA (12 to 18 S) was converted to corresponding single stranded complementary DNA (cDNA) from which was produced double stranded cDNA. After poly-dC tailing, it was inserted into a vector, such as a plasmid bearing one or more phenotypic markers.

5. The thus prepared vectors were used to transform bacterial cells providing a colony library. Radiolabeled cDNA prepared from both induced and uninduced mRNA, derived as described above, was used to separately probe duplicate colony libraries. The excess cDNa was then removed and the colonies exposed to X-ray film so as to identify the induced cDNA clones.

6. From the induced cDNA clones the corresponding plasmid DNA was isolated and sequenced.

7. In a first embodiment sequenced DNA was than tailored *in vitro* for insertion into an appropriate

expression vehicle which was used to transform E. coli host cell which was, in turn, permitted to grow in a culture and to express the desired human immune interferon product.

8. Human immune interferon thus expressed doubtless has 146 amino acids in its mature form, beginning with cysteine, and is very basic in character. Its monomeric molecular weight has been calculated at 17,140. Perhaps because of the presence of numerous basic residues, hydrophobicity, salt bridge formation and so forth, the molecule may associate itself in oligomeric forms, e.g., in dimer, trimer or tetramer form. The high molecular weights previously observed with natural material (6) which can not be accounted for on the basis of the amino acid sequence alone may be due to such oligomeric forms as well as to the contribution of carbohydrate from post-translational glycosylation.

9. In certain host cell systems, particularly when ligated into an expression vehicle so as to be expressed together with its signal peptide, the mature form of human immune interferon is exported into the cell culture medium, immeasurably aiding in recovery and purification methods.

Description of preferred embodiments

A. Microorganisms/cell cultures

1. Bacterials strains/promoters

The work described herein was performed employing, *inter alia*, the microorganism *E. coli* K-12 strain 294 (end A, thi$^-$, hsr$^-$, $_k$hsm$^+$), as described in British Patent Publication No. 2055382A. This strain has been deposited with the American Type Culture Collection, ATCC Accession No. 31446. However, various other microbial strains are useful, including known *E. coli* strains such as *E. coli* B, *E. coli* X 1776 (ATCC No. 31537) and *E. coli* W 3110 (F$^-$, $\lambda^-$, protrophic) ATCC No. 27325), or other microbial strains many of which are deposited and (potentially) available from recognized microorganism depository institutions, such as the American Type Culture Collection (ATCC)—cf. the ATCC catalogue listing. See also German Offenlegungsschrift 2644432. These other microorganisms include, for example, *Bacilli* such as *Bacillus subtilis* and other enterobacteriaceae among which can be mentioned as examples *Salmonella typhimurium* and *Serratia marcesans*, utilizing plasmids that can replicate and express heterologous gene sequences therein.

As examples, the beta lactamase and lactose promoter systems have been advantageously used to initiate and sustain microbial production of heterologous polypeptides. Details relating to the make-up and construction of these promoter systems have been published by Chang *et al., Nature 275*, 617 (1978) and Itakura *et al., Science 198*, 1056 (1977), which are hereby incorporated by reference. More recently, a system based upon tryptophan, the so-called *trp* promoter system, has been developed. Details relating to the make-up and construction of this system have been published by Goeddel *et al., Nucleic Acids Research 8*, 4057 (1980) and Kleid *et al.*, U.S.S.N. 133, 296, filed March 24, 1980, which are hereby incorporated by reference. Numerous other microbial promoters have been discovered and utilized and details concerning their nucleotide sequences, enabling a skilled worker to ligate them functionally within plasmid vectors, have been published—see, e.g., Siebenlist *et al., Cell 20*, 269 (1980), which is incorporated herein by this reference.

2. Yeast strains/yeast promoters

The expression system hereof may also employ the plasmid YRp7 (14, 15, 16), which is capable of selection and replication in both *E. coli* and the yeast, *Saccharomyces cerevisiae*. For selection in yeast the plasmid contains the *TRP1* gene (14, 15, 16) which complements (allows for growth in the absence of tryptophan) yeast containing mutations in this gene found on chromosone IV of yeast (17). The strain used here was the strain RH218 (18) deposited at the American Type Culture Collection without restriction (ATCC No. 44076). However, it will be understood that any *Saccharomyces cerevisiae* strain containing a mutation which makes the cell trp1 should be an effective environment for expression of the plasmid containing the expression system. An example of another strain which could be used is *pep4-1* (19). This tryptophan auxotroph strain also has a point mutation in *TRP*1 gene.

When placed on the 5′ side of a non-yeast gene the 5′-flanking DNA sequence (promoter) from a yeast gene (for alcohol dehydrogenase 1) can promote the expression of a foreign gene in yeast when placed in a plasmid used to transform yeast. Besides a promoter, a proper expression of a non-yeast gene in yeast requires a second yeast sequence placed at the 3′-end of the non-yeast gene on the plasmid so as to allow for proper transcription termination and polyadenylation in yeast. This promoter can be suitably employed in the present invention as well as others—see *infra*. In the preferred embodiments, the 5′-flanking sequence of the yeast 3-phosphoglycerate kinase gene (20) is placed upstream from the structural gene followed again by DNA containing termination—polyadenylation signals, for example, the *TRP*1 (14, 15, 16) gene or the PGK (20) gene.

Because yeast 5′-flanking sequence (in conjunction with 3′- yeast termination DNA) (*infra*) can function to promote expression of foreign genes in yeast, it seems likely that the 5′-flanking sequences of any highly-expressed yeast gene could be used for the expression of important gene products. Since under some circumstances yeast expressed up to 65 percent of its soluble protein as glycolytic enzymes (21) and since this high level appears to result from the production of high levels of the individual mRNAs (22), it should be possible to use the 5′-flanking sequences of any other glycolytic genes for such expression purposes—e.g., enolase, glyceraldehyde - 3 - phosphate dehydrogenase, hexokinase, pyruvate

decarboxylase, phosphofructokinase, glucose - 6 - phosphate isomerase, 3 - phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Any of the 3'-flanking sequences of these genes could also be used for proper termination and mRNA polyadenylation in such an expression system—cf. *Supra*. Some other highly expressed genes are those for the acid phosphatases (23) and those that express high levels of production due to mutations in the 5'-flanking regions (mutants that increase expression)—usually due to the presence of a TY1 transposable element (24).

All of the genes mentioned above are thought to be transcribed by yeast RNA polymerase II (24). It is possible that the promoters for RNA polymerase I and III which transcribe genes for ribosomal RNA, 5S RNA, and tRNAs (24, 25), may also be useful in such expression constructions.

Finally, many yeast promoters also contain transcriptional control so they may be turned off or on by variation in growth conditions. Some examples of such yeast promoters are the genes that produce the following proteins: Alcohol dehydrogenase II, isocytochrome-c, acid phosphatase, degradative enzymes associated with nitrogen metabolism, glyceraldehyde - 3 - phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization (22). Such a control region would be very useful in controlling expression of protein product—especially when their production is toxic to yeast. It should also be possible to put the control region of one 5'-flanking sequence with a 5'-flanking sequence containing a promoter from a highly expressed gene. This would result in a hybrid promoter and should be possible since the control region and the promoter appear to be physically distinct DNA sequences.

3. Cell culture systems/cell culture vectors

Propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (see Tissue Culture, Academic Press, Kruse and Patterson eds. 1973). Employed herein was the COS-7 line of monkey kidney fibroblasts as the host for the production of immune interferon (25a). However, the experiments detailed here could be performed in any cell line which is capable of the replication and expression of a compatible vector, e.g., WI38, BHK, 3T3, CHO, VERO, and HeLa cell lines. Additionally, what is required of the expression vector is an origin of replication and a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences. While these essential elements of SV40 have been exploited herein, it will be understood that the invention, although described herein in terms of a preferred embodiment, should not be construed as limited to these sequences. For example, the origin of replication of other viral (e.g., Polyoma, Adeno, VSV, BPV, and so forth) vectors could be used, as well as cellular origins of DNA replication which could function in a nonintegrated state.

B. Vector systems

1. Direct expression of mature immune interferon in *E. coli*

The procedure used to obtain direct expression of IFN-γ in *E. coli* as a mature interferon polypeptide (minus signal sequence) was a variant of that employed earlier for human growth hormone (26) and human leukocyte interferon (1), insofar as it involved the combination of synthetic (N-terminal) and cDNAs.

As deduced from the nucleotide sequence of p69, described *infra*, and by comparison with the known cleavage site between signal peptide and mature polypeptide for several IFN-αs (2), IFN-γ has a hydrophobic signal peptide of 20 amino acids followed by 146 amino acids of mature IFN-γ (Figure 5). As shown in Figure 7, a *BstN*I restriction endonuclease site is conveniently located at amino acid 4 of mature IFN-γ. Two synthetic oligodeoxynucleotides were designed which incorporate an ATG translational initiation codon, codons for amino acids 1, 2 and 3 (cysteine - tyrosine - cysteine) and create an *Eco*RI cohesive end. These deoxyoligonucleotides were ligated to a 100 base pair *BstN*I-*Pst*I fragment of p69 to construct a 1115 base pair synthetic-natural hybrid gene which codes for IFN-γ and which is bounded by *Eco*RI and *Pst*I restriction sites. This gene was inserted into the plasmid pLeIF A trp 103 between the *Eco*RI and *Pst*I sites to give the expression plasmid pIFN-γ trp 48. In this plasmid the IFN-γ gene is expressed under the control of the *E. coli* trp promoter. (pLeIF A trp 103 is a derivative of pLeIF A 25 in which the *Eco*RI site distal to the LeIF A gene was removed. The procedure used to remove this *Eco*RI site has been described previously (27)).

2. Expression in yeast

To express a heterologous gene such as the cDNA for immune interferon in yeast, it was necessary to construct a plasmid vector containing four components. The first component is the part which allows for transformation of both *E. coli* and yeast and thus must contain a selectable gene from each organism. (In this case, this is the gene for ampicillin resistance from *E. coli* and the gene *TRP*1 from yeast). This component also requires an origin of replication from both organisms to be maintained as a plasmid DNA in both organisms. (In this case, this is the *E. coli* origin from pBR322 and the *ars*1 origin from chromosone III of yeast).

The second component of the plasmid is a 5'-flanking sequence from a highly expressed yeast gene to promote transcription of a downstream-placed structural gene. In this case, the 5'-flanking sequence used is that from the yeast 3 - phosphoglycerate kinase (PGK) gene. The fragment was constructed in such a way so as to remove the ATG of the PGK structural sequence as well as 8 bp upstream from this ATG. This

sequence was replaced with a sequence containing both an *Xba*I and *Eco*RI restriction site for convenient attachment of this 5'-flanking sequence to the structural gene.

The third component of the system is a structural gene constructed in such a manner that it contains both an ATG translational start and translational stop signals. The isolation and construction of such a gene is described *infra*.

The fourth component is a yeast DNA sequence containing the 3'-flanking sequence of a yeast gene, which contains the proper signals for transcription termination and polyadenylation.

With all these components present, immune interferon has been produced in yeast.

3. Expression in mammalian cell culture

The strategy for the synthesis of immune interferon in mammalian cell culture relied on the development of a vector capable of both autonomous replication and expression of a foreign gene under the control of a heterologous transcriptional unit. The replication of this vector in tissue culture was accomplished by providing a DNA replication origin (derived from SV40 virus), and providing helper function (T antigen) by the introduction of the vector into a cell line endogenously expressing this antigen (28, 29). The late promoter of SV40 virus preceded the structural gene of interferon and ensured the transcription of the gene.

The vector used to obtain expression of IFN-γ consisted of pBR322 sequences which provided a selectable marker for selection in *E. coli* (ampicillin resistance) as well as an *E. coli* origin of DNA replication. These sequences were derived from the plasmid pML-1 (28) and encompassed the region spanning the *Eco*RI and *Bam*HI restriction sites. The SV40 origin is derived from a 342 base pair *Pvu*II-*Hind*III fragment encompassing this region (30, 31) (both ends being converted to *Eco*RI ends). These sequences, in addition to comprising the viral origin of DNA replication, encode the promoter for both the early and late transcriptional unit. The orientation of the SV40 origin region was such that the promoter for the late transcriptional unit was positioned proximal to the gene encoding interferon.

Brief description of the drawings

Figure 1 depicts a sucrose gradient centrifugation of induced Peripheral Blood Lymphocyte (PBL) Poly(A)+RNA. Two peaks of interferon activity were observed (as shown by the hatched boxes) with sizes of 12S and 16S. The positions of ribosomal RNA markers (centrifuged independently) are labelled above the absorbance profile.

Figure 2 depicts an electrophoresis of induced PBL Poly(A)+RNA through an acid-urea-agarose. Only one peak of activity was observed, which comigrated with 18S RNA. The positions of ribosomal RNA markers which were electrophoresed in an adjacent lane and visualized by ethidium bromide staining are labelled above the activity profile.

Figure 3 shows hybridization patterns of 97 colonies with induced and uninduced $^{32}$P-labelled cDNA probes. 96 individual transformants were grown in a microtiter plate, replica plated on two nitrocellulose membranes, and then the filters were hybridized with $^{32}$P-cDNA probes prepared from either induced mRNA (above) or mRNA isolated from uninduced PBL cultures (uninduced, below). The filters were washed to remove non-hybridized RNA and then exposed to X-ray film. This set of filters is representative of 86 such sets (8300 independent colonies). An example of an "induced" clone is labelled as H12.

Figure 4 is a restriction endonuclease map of the clone 69 cDNA insert. The lower cDNA insert is bounded by *Pst*I sites (dots at both ends) and oligo dC-dG tails (single lines). The number and size of fragments produced by restriction nuclease cleavage was estimated by electrophoresis through 6 percent acrylamide gels. Positions of sites was confirmed by nucleic acid sequencing (presented in Figure 5). The coding region of the largest open reading frame is boxed and the hatched region represents the putative 20 residue signal peptide sequence, while the stipled region represents the mature IIF sequence (146 amino acids). The 5' end of the mRNA is to the left while the 3' end is to the right.

Figure 5 illustrates the nucleotide sequence of the plasmid p69 cDNA insert, however, illustrating the most common allelic form of IFN-γ. The deduced amino acid sequence of the longest open reading frame is also presented. The putative signal sequence is represented by the residues labelled S1 to S20.

Figure 6 is a comparison of IFN-γ mRNA structure with that of leukocyte (IFN-α) and fibroblast (IFN-β) interferons. The clone 69 mRNA (labelled immune) contains significantly greater amounts of untranslated sequences.

Figure 7 is a schematic diagram of the construction of the IFN-γ expression plasmid pIFN-γ trp 48. The starting material is the 1250 base pair *Pst*I cDNA insert from plasmid p69.

Figure 8 shows a diagram of plasmid used for expression of IFN-γ in monkey cells.

Figure 9 depicts a Southern hybridization of eight different *Eco*RI digested human genomic DNAs hybridized with $^{32}$P-labelled 600 base pair *Dde*I fragment from the cDNA insert of p69. Two *Eco*RI fragments clearly hybridize with the probe in each DNA sample.

Figure 10 depicts a Southern hybridization of human genomic DNA digested with six different restriction endonucleases hybridized with the $^{32}$P-labelled probe from p69.

Figure 11 schematically illustrates the restriction map of the 3.1 kbp *Hind*III insert of vector pB1 from which the PGK promoter was isolated. Indicated is the insertion of an *Eco*RI site and an *Xba*I site in the 5'-flanking DNA of the PGK gene.

Figure 12 illustrates the 5'-flanking sequence plus the initial coding sequence for the PGK gene before insertion of an *Xba*I and *Eco*RI sites.

Figure 13 schematically illustrates techniques used to insert an *Xba*I site at position—8 in the PGK promoter and to isolate a 39bp fragment of the 5'-flanking sequence of *PGK* containing this *Xba*I end and a *Sau*3A end.

Figure 14 schematically illustrates the construction of a 300 bp fragment containing the above 39bp fragment, additional *PGK* 5'-flanking sequence (265bp) from *Pvu*I to *Sau*3A (see Figure 11), and a *Eco*RI site adjacent to *Xba*I.

Figure 15 schematically illustrates the construction of the 1500 bp PGK promoter fragment (*Hind*III/*Eco*RI) which contains, in addition to the fragment constructed in Figure 14, a 1300bp *Hind*III to *Pvu*I fragment from *PGK* 5'-flanking sequence (see Figure 11).

Figure 16 illustrates the composition of an expression vector for human immune interferon in yeast, containing the modified PGK promoter, the IFN-γ cDNA and the terminator region of the yeast PGK gene as described in more detail herein.

Detailed description

A. Source of IFN-γ mRNA

Peripheral Blood Lymphocytes (PBLs) were derived from human donors by leukophoresis. PBLs were further purified by Ficoll-Hypaque gradient centrifugation and then cultured at a concentration of $5\times10^6$ cells/ml in RPMI 1640, 1 percent L-glutamine, 25 mM HEPES, and 1 percent penicillin-streptomycin solution (Gibco, Grand Island, NY). These cells were induced to produce IFN-γ by the mitogen staphlococcal enterotoxin B (1 µg/ml) and cultured for 24 to 48 hours at 37°C in 5 percent $CO_2$. Desacetylthymosin - α - 1 (0.1 µg/ml) was added to PBL cultures to increase the relative yield of IFN-γ activity.

B. Messenger RNA isolation

Total RNA from PBL cultures was extracted essentially as reported by Berger, S. L. *et al.* (33). Cells were pelleted by centrifugation and then resuspended in 10 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$ and 10 mM ribonucleoside vanadyl complex. Cells were lysed by the addition of NP-40 (1 percent final concentration), and nuclei were pelleted by centrifugation. The supernatant contained the total RNA which was further purified by multiple phenol and chloroform extractions. The aqueous phase was made 0.2 M in NaCl and then total RNA was precipitated by the addition of two volumes of ethanol. RNA from uninduced (nonstimulated) cultures was isolated by the same methods. Oligo-dT cellulose chromatography was utilized to purify mRNA from the total RNA preparations (34). Typical yields from 1—2 liters of cultured PBLs were 5—10 milligrams of total RNA and 50—200 micrograms of Poly(A)+RNA.

C. Size fractionation of mRNA

Two methods were used to fractionate mRNA preparations. These methods were used independently (rather than in unison) and each resulted in a significant enrichment of IFN-γ mRNA.

Sucrose gradient centrifugation in the presence of the denaturant formamide was used to fractionate mRNA. Gradients of 5 percent to 25 percent sucrose in 70 percent formamide (32) were centrifuged at 154,000×g for 19 hours at 20°C. Successive fractions (0.5 ml) were then removed from the top of the gradient, ethanol precipitated, and an aliquot was injected into *Xenopus laevis* oocytes for translation of the mRNA (35). After 24 hrs at room temperature, the incubation medium was then assayed for antiviral activity in a standard cytopathic effect inhibition assay employing Vesicular Stomatitis Virus (Indiana strain) or Encephalomyocarditis Virus on WISH (human amnion) cells as described by Stewart (36), except that the samples were incubated with the cells for 24 hours (instead of 4) prior to challenge with the virus. Two activity peaks were consistently observed in sucrose gradient fractionated RNA (Figure 1). One peak sedimented with a calculated size of 12S and contained 100—400 units/ml of antiviral activity (compared with a IFN-α standard) per microgram of RNA injected. The other peak of activity sedimented as 16S in size and contained about half the activity of the slower sedimenting peak. Each of these activity peaks appears to be due to IFN-γ, since no activity was observed when the same fractions were assayed on a bovine cell link (MDBK) which is not protected by human IFN-γ. Both IFN-α activity and IFN-β activity would have been easily detected with the MDBK assay (5).

Fractionation of mRNA (200 µg) was also performed by electrophoresis through acid urea agarose gels. The slab agarose gel (37, 38) was composed of 1.75 percent agarose, 0.025 M sodium citrate, pH 3.8 and 6 M urea. Electrophoresis was performed for 7 hours at 25 milliamp and 4°C. The gel was then fractionated with a razor blade. The individual slices were melted at 70°C and extracted twice with phenol and once with chloroform. Fractions were then ethanol precipitated and subsequently assayed for IFN-γ mRNA by injection into *Xenopus laevis* oocytes and antiviral assay. Only one peak of activity was observed in gel fractionated samples (Figure 2). This peak comigrated with 18S RNA and had an activity of 600 units/ml per microgram of injected RNA. This activity also appeared to be IFN-γ specific, since it did not protect MDBK cells.

The size discrepancy between activity peaks observed on sucrose gradients (12S and 16S) and acid urea gels (18S) may be explained by the observation that these independent fractionation methods are not performed under total denaturing conditions.

D. Preparation of a colony library containing IFN-γ sequences

3 µg of gel-fractionated mRNA was used for the preparation of double stranded cDNA by standard procedures (26, 39). The cDNA was size fractionated on a 6 percent polyacrylamide gel. Two size fractions were electroeluted, 800—1500 bp (138 ng) and >1500 bp (204 ng). 35 ng portions of each size cDNA was extended with deoxyC residues using terminal deoxynucleotidyl transferase (40) and annealed with 300 ng of the plasmid pBR322 (41) which had been similarly tailed with deoxyG residues at the *Pst*I site (40). Each annealed mixture was then transformed into *E. coli* K12 strain 294. Approximately 8000 transformants were obtained with the 800—1500 pb cDNA and 400 transformants were obtained with the >1500 bp cDNA.

E. Screening of colony library for induced cDNAs

The colonies were individually inoculated into wells of microtitre plates containing LB (58)+5 µg/ml tetracycline and stored at −20°C after addition of DMSO to 7 percent. Two copies of the colony library were grown up on nitrocellulose filters and the DNA from each colony fixed to the filter by the Grunstein-Hogness procedure (42).

$^{32}$P-labelled cDNA probes were prepared using 18S size gel fractionated mRNA from induced and uninduced PBL cultures. Oligo $dT_{12-18}$ was the primer used and reaction conditions have been previously described (1). Filters containing 8000 transformants from the 600—1500 bp cDNA size cut and 400 transformants from the >1500 bp cDNA size cut were hybridized with $20\times10^6$ cpm of induced $^{32}$P-cDNA. A duplicate set of filters was hybridized with $20\times10^6$ cpm of uninduced $^{32}$P-cDNA. Hybridization was for 16 hours using conditions described by Fritsch *et al.* (43). Filters were extensively washed (43) and then exposed to Kodak XR-5 X-ray film with DuPont Lightning-Plus intensifying screens for 16—48 hours. Each colony's hybridization pattern with the two probes was compared. Approximately 40 percent of the colonies clearly hybridized with both probes, while approximately 50 percent of the colonies failed to hybridize with either probe (presented in Figure 3). 124 colonies hybridized significantly with the induced probe but undetectably or more weakly with the uninduced probe. These colonies were individually inoculated into wells of microtitre plates, grown and transferred to nitrocellulose filters, and hybridized with the same two probes, as described above. Plasmid DNA isolated from each of these colonies by a rapid method (44) was also bound to nitrocellulose filters and hybridized (45) with the induced and uninduced probes. DNA from 22 colonies hybridized with only the induced probe and were termed "induced" colonies.

F. Characterization of induced colonies

Plasmid DNA was prepared from 5 of the induced colonies (46) and used for characterization of the cDNA inserts. Restriction endonuclease mapping of five induced plasmids (p67, p68, p69, p71 and p72) suggested that four had similar restriction nuclease maps. These four (p67, p69, p71 and p72) each had four *Dde*I sites, 2 *Hinf*I sites, and a single *Rsa*I site in the cDNA insert. The fifth plasmid (p68) contained a common *Dde*I fragment and appeared to be a short cDNA clone related to the other four. The homology suggested by restriction nuclease mapping was confirmed by hybridization. A $^{32}$P-labelled DNA probe was prepared (47) from a 600 bp *Dde*I fragment of the p67 plasmid and used for hybridization (42) to the other induced colonies. All five of the restriction nuclease mapped colonies cross-hybridized with this probe, as did 17 other colonies of the 124 chosen in the induced/uninduced screening. The length of cDNA insert in each of these cross-hybridizing plasmids was determined by *Pst*I digestion and gel electrophoresis. The clone with the longest cDNA insert appeared to be clone 69 with an insert length of 1200—1400 bp. This DNA was used for all further experiments, and its restriction endonuclease map is shown in Figure 4.

The cDNA insert in p69 was demonstrated to be IFN-γ cDNA by its expression products, produced in three independent expression systems, yielding antiviral activity, as described in more detail *infra*.

G. Sequence analysis of cDNA insert of p69

The complete nucleotide sequence of the plasmid p69 cDNA insert was determined by the dideoxynucleotide chain termination method (48) after subcloning fragments into the M13 vector mp7 (49) and by the Maxam-Gilbert chemical procedure (52). The longest open reading frame encodes a protein of 166 amino acids, presented in Figure 5. The first residue encoded is the first met codon encountered in the 5' end of the cDNA. The first 20 residues at the amino terminus probably serves as a signal sequence for the secretion of the remaining 146 amino acids. This putative signal sequence has features in common with other characterized signal sequences such as size and hydrophobicity. Furthermore, the four amino acids found at the putative cleavage sequence (ser-leu-gly-cys) are identical with four residues found at the cleavage point of several leukocyte interferons (LeIF B, C, D, F, and H, (2)). The encoded mature amino acid sequence of 146 amino acids (hereinafter referred to as "recombinant human immune interferon") has a molecular weight of 17,140.

There are two potential glycosylation positions (50) in the encoded protein sequence, at amino acids 28 to 30 (asn-gly-thr) and amino acids 100 to 102 (asn-tyr-ser). The existence of these positions is consistent with the observed glycosylation of human IFN-γ (6, 51). In addition, the only two cysteine residues (positions 1 and 3) are sterically too close to form a disulfide bridge, which is consistent with the observed stability of IFN-γ in the presence of reducing agents such as β-mercaptoethanol (51). The deduced mature

amino acid sequence is generally quite basic, with 30 total lysine, arginine, and histidine residues and only 19 total aspartic acid and glutamic acid residues.

The mRNA structure of IFN-γ as deduced from DNA sequence of plasmid p69 is distinctively different from IFN-α (1, 2) or IFN-β (5) mRNA. As presented in Figure 6, the coding region of IFN-γ is shorter while the 5' untranslated and 3' untranslated regions are much longer than either IFN-α or IFN-β.

H. Expression of recombinant human immune interferon in *E. coli*

With reference to Figure 7, 50 µg of plasmid p69 were digested with *Pst*I and the 1250 base pair insert isolated by gel electrophoresis on a 6 percent polyacrylamide gel. Approximately 10 µg of this insert was electroeluted from the gel. 5 µg of this *Pst*I fragment was partially digested with 3 units of *BstN*I (Bethesda Research Labs) for 15 minutes at 37°C and the reaction mixture purified on a 6 percent polyacrylamide gel. Approximately 0.5 µg of the desired 1100 base pair *BstN*I-*Pst*I fragment was recovered. The two indicated deoxyoligonucleotides, 5'-dAATTCATGTGTTATTGTC and 5'-dTGACAATAACACATG (Figure 7) were synthesized by the phosphotriester method (53) and phosphorylated as follows. 100 pmoles of eachy deoxyoligonucleotide were combined in 30 µl of 60 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 15 mM β-mercaptoethanol and 240 µCi ($\gamma$-$^{32}$P)ATP (Amersham, 5000 Ci/mmole). 12 units of T4 polynucleotide kinase were added and the reaction allowed to proceed at 37°C for 10 minutes. 1 µl of 10 mM ATP was added and the reaction allowed to proceed an additional 20 minutes. After ϕ-OH/$CHCl_3$ extraction the oligomers were combined with 0.25 µg of the *BstN*I-*Pst*I 1100 base pair fragment and ethanol precipitated. These fragments were ligated at 20°C for 2 hours in 30 µl of 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM ATP and 10 units T4 DNA ligase. The mixture was digested for 1 hour with 30 units of *Pst*I and 30 units of *Eco*RI (to eliminate polymerization through ligation of cohesive termini) and electrophoresed on a 6 percent polyacrylamide gel. The 1115 base pair product (110,000 cpm) was recovered by electroelution.

The plasmid pLeIF A trp 103 (Figure 7) is a derivative of the plasmid pLeIF A 25 (1) in which the *Eco*RI site distal to the LeIF A gene has been removed (27). 3 µg of pLeIF A trp 103 was digested with 20 units of *Eco*RI and 20 units of *Pst*I for 90 minutes at 37°C and electrophoresed on a 6 percent polyacrylamide gel. The large (~3900 base pair) vector fragment was recovered by electroelution. The 1115 base pair *Eco*Ri-*Pst*I IFN-γ DNA fragment was ligated into 0.15 µg of this prepared vector. Transformation of *E. coli* K-12 strain 294 (ATCC No. 31446) gave 120 tetracycline resistant colonies. Plasmid DNA was prepared from 60 of these transformants and digested with *Eco*RI and *Pst*I. Three of these plasmids contained the desired 1115 base pair *Eco*RI-*Pst*I fragment. DNA sequence analysis verified that these plasmids had the desired nucleotide sequence at the junctions between the *trp* promoter, synthetic DNA and cDNA. One of these plasmids pIFN-γ trp 48 was chosen for additional study. This plasmid was used to transform the *E. coli* K-12 strain W3110 (ATCC No. 27325).

I. Gene structure of the IFN-γ coding sequence

The structure of the gene coding for IFN-γ was analyzed by Southern hybridization. In this procedure (54), 5 micrograms of high molecular weight human lymphocyte DNA (prepared as in 55) is digested to completion with various restriction endonucleases, electrophoresed on 1.0 percent agarose gels (56), and blotted to a nitrocellulose filter (54). A $^{32}$P-labelled DNA probe was prepared (47) from a 600 bp *Dde*I fragment of the cDNA insert of p69 and hybridized (43) with the nitrocellulose-DNA blot. $10^7$ counts per minute of the probe were hybridized for 16 hours and then washed as described (43). Eight genomic DNA samples from different human donors were digested with the *Eco*RI restriction endonuclease and hybridized with the p69 $^{32}$P-labelled probe. As presented in Figure 9, two clear hybridization signals are observed with sizes of 8.8 kilobase pairs (kbp) and 2.0 kbp as estimated by comparison of mobilities with *Hind*III digested λDNA. This could be the result of two IFN-γ genes or a single gene split by an *Eco*RI site. Since the p69 cDNA contains no *Eco*RI site, an intervening sequence (intron) with an internal *Eco*RI site would be necessary to explain a single gene. To distinguish between these possibilities, another Southern hybridization was performed with the same probe against five other endonuclease digestions of a single human DNA (Figure 10). Two hybridizing DNA fragments were observed with two other endonuclease digests, *Pvu*II (6.7 kbp and 4.0 kbp) and *Hinc*II (2.5 kbp and 2.2 kbp). However, three endonuclease digestion patterns provide only a single hybridizing DNA fragment: *Hind*III (9.0 kbp), *Bgl*II (11.5 kbp) and *Bam*HI (9.5 kbp). Two IFN-γ genes would have to be linked at an unusually close distance (less than 9.0 kbp) to be contained within the same *Hind*III hybridizing fragment. This result suggests that only a single homologous IFN-γ gene (unlike the many related IFN-α genes) is present in human genomic DNA and that this gene is split by one or more introns containing *Eco*RI, *Pvu*II, and *Hinc*II sites. This prediction was supported by hybridization of a $^{32}$P-labelled (47) fragment prepared from just the 3' untranslated region of the cDNA from p69 (130 bp *Dde*I fragment from 860 bp to 990 bp in Figure 5) against an *Eco*RI digest of human genomic DNA. Only the 2.0 kbp *Eco*RI fragment hybridized to this probe, suggesting that this fragment contains the 3' untranslated sequences, while the 8.8 kbp *Eco*RI fragment contains the 5' sequences. The gene structure of IFN-γ (one gene with at least one intron) is distinctly different from IFN-α (multiple genes (2) without introns (56)) or IFN-β (one gene with no introns (57)).

J. Preparation of bacterial extracts

An overnight culture of *E. coli* W3110/pIFN-γ trp 48 in Luria broth+5 micrograms per ml tetracycline was used to inoculate M9 (58) medium containing 0.2 percent glucose, 0.5 percent casamino acids, and 5

micrograms per ml tetracycline at a 1:100 dilution. Indole acrylic acid was added to a final concentration fo 20 micrograms per ml when $A_{550}$ was between 0.1 and 0.2. Ten ml samples were harvested by centrifugation at $A_{550}$=1.0 and resuspended immediately in 1 ml phosphate buffered saline containing 1 mg per ml bovine serum albumin (PBS-BSA). Cells were opened by sonication and cleared of debris by centrifugation. The supernatants were stored at 4°C until assay. Interferon activity in the supernatants was determined to be 250 units/ml by comparison with IFN-α standards by the cytopathic effect (CPE) inhibition assay.

K. Transformation of yeast/strains and media

Yeast strains were transformed as previously described (59). *E. coli* strain JA300 (*thr leu*B6 *thi thyA trp*C1117 *hsdm*$^-$ *hsd*R$^-$ str$^R$) (20) was used to select for plasmids containing functional *TRP*1 gene. Yeast strain RH218 having the genotype (a *trp*1 *gal2 SUC2 mal* CUP1) (18) was used as yeast transformation host. RH218 has been deposited without restriction in the American Type Culture Collection, ATCC No. 44076. M9 (minimal medium) with 0.25 percent casamino acids (CAA) and LB (rich medium) were as described by Miller (58) with the addition of 20 µg/ml ampicillin (Sigma) after media is autoclaved and cooled. Yeast were grown on the following media: YEPD contained 1 percent yeast extract, 2 percent peptone and 2 percent glucose ±3 percent Difco agar. YNB+CAA contained 6.7 grams of yeast nitrogen base (without amino acids) (YNB) (Difco), 10 mg of adenine, 10 mg of uracil, 5 grams CAA, 20 grams glucose and ±30 grams agar per liter.

L. Construction of yeast expression vector

1. 10 µg of YRp7 (14, 15, 16) was digested with *Eco*RI. Resulting sticky DNA ends were made blunt using DNA Polymerase I (Klenow fragment). Vector and insert were run on 1 percent agarose (SeaKem) gel, cut from the gel, electroeluted and extracted 2× with equal volumes of chloroform and phenol before precipitation with ethanol. The resulting blunt end DNA molecules were then ligated together in a final volume of 50 µl for 12 hours at 12°C. This ligation mix was then used to transform *E. coli* strain JA300 to ampicillin resistance and tryptophan prototrophy. Plasmids containing the *TRP*1 gene in both orientations were isolated. pFRW1 had the *TRP*1 gene in the same orientation as YRp7 while pFRW2 had the *TRP*1 gene in the opposite orientation.

20 µg of pFRW2 was linearized with *Hind*III and electrophoresed on a 1 percent agarose gel. Linear molecules were eluted from the gel and 200 ng were then ligated with 500 ng of the 3.1 kb *Hind*III insert of plasmid pB1 (13) which is a restriction fragment containing the yeast 3-phosphoglycerate kinase gene. The ligation mix was used to transform *E. coli* strain 294 to ampicillin resistance and tetracycline sensitivity. Plasmid prepared from one such recombinant had an intact *TRP*1 gene with the 3.1 kbp *Hind*III fragment from pB1 insert DNA in the *Hind*III site of the tetracycline resistance gene. This plasmid is pFRM31. 5 µg of pFRM31 was completely digested with *Eco*RI, extracted twice with phenol and chloroform then ethanol precipitated. The cohesive ends of the molecule were filled in using DNA Polymerase I (Klenow fragment) in a reaction which was made 250 µM in each deoxynucleoside triphosphate. The reaction was performed for 20 minutes at 14°C at which time the DNA was extracted two times with phenol-chloroform, and then precipitated with ethanol. The resuspended DNA was then completely digested with *Cla*I and electrophoresed on a 6 percent acrylamide gel. The vector fragment was eluted from the gel, phenol-chloroform extracted and ethanol precipitated.

The six N-terminal amino acids of the 3-phosphoglycerate enzyme purified from humans are as follows:

| 1 | — | 2 | — | 3 | — | 4 | — | 5 | — | 6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SER | — | LEU | — | SER | — | ASN | — | LYS | — | LEU | — |

One of the translational reading frames generated from the DNA sequence of the 141 bp *Sau3A*-to-*Sau*3A restriction fragment (containing the internal *Hinc*III site; see *PGK* restriction map Figure 11) produces the following amino acid sequence.

| | | 1 | — | 2 | — | 3 | — | 4 | — | 5 | — | 6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MET | — | SER | — | LEU | — | SER | — | SER | — | LYS | — | LEU | — |

After removal of initiator methionine, it is seen that PGK N-terminal amino acid sequence has 5 of 6 amino acid homology with N-terminal amino acid sequence of human PGK.

This sequencing result suggested that the start of the yeast PGK structural gene is coded for by DNA in the 141bp *Sau*3A restriction fragment of pB1. Previous work (20) has suggested that the DNA sequences specifying the PGK mRNA may reside in this area of the HindIII fragment. Further sequencing of the 141 bp *Sau*3A fragment gives more DNA sequence of the PGK promoter (Figure 12).

A synthetic oligonucleotide with the sequence 5'-ATTTGTTGTAAA3' was synthesized by standard methods (Crea *et al., Nucleic Acids Res.* 8, 2331 (1980)). 100 ng of this primer was labelled at the 5' end using 10 units of T4 polynucleotide kinase in a 20 µl reaction also containing 200 µCi of [$\gamma^{32}$-P] ATP. This labelled primer solution was used in a primer-repair reaction designed to be the first step in a multi-step

process to put an *Eco*RI restriction site in the *PGK* 5'-flanking DNA just preceding PGK structure gene sequence.

100 µg of pB1 (20) was completely digested with *Hae*III then run on a 6 percent polyacrylamide gel. The uppermost band on the ethidum stained gel (containing PGK promoter region) was isolated by electroelution as described above. This 1200 bp *Hae*III piece of DNA was restricted with *Hin*cII then run on a 6 percent acrylamide gel. The 650 bp band was isolated by electroelution. 5 µg of DNA was isolated. This 650 bp *Hae*III-to-*Hin*cII piece of DNA was resuspended in 20 µl $H_2O$, then mixed with the 20 µl of the phosphorylated primer solution described above. This mixture was 1× phenol-chloroform extracted then ethanol precipitated. Dried DNA was resuspended in 50 µl of $H_2O$ and then heated in a boiling water bath for seven minutes. This solution was then quickly chilled in a dry ice-ethanol bath (10—20 seconds) then transferred to an ice-water bath. To this solution was added 50 µl of a solution containing 10 µl of 10× DNA polymerase I buffer (Boehringer Mannheim), 10 µl of a solution previously made 2.5 mM in each deoxynucleoside triphosphate (dATP, dTTP, dGTP and dCTP), 25 µl of $H_2O$ and 5 units of DNA Polymerase I, Klenow fragment. This 100 µl reaction was incubated at 37°C for 4 hours. The solution was then 1× phenol-chloroform extracted, ethanol precipitated, dried by lyophilization then exhaustively restricted with 10 units of *Sau*3A. This solution was then run on a 6 percent acrylamide gel. The band corresponding to 39 bp in size was cut from the gel then isolated by electroelution described above. This 39 bp band has one blunt end and one *Sau*3A sticky end. This fragment was cloned into a modified pFIF trp 69 vector (5). 10 µg of pFIF trp 69 was linearized with *Xba*I, 1× phenol chloroform extracted, then ethanol precipitated. The *Xba*I sticky end was filled in using DNA Polymerase I Klenow fragment in a 50 µl reaction containing 250 µM in each nucleoside triphosphate. This DNA was cut with *Bam*HI then run a 6 percent acrylamide gel. The vector fragment was isolated from the gel by electroelution then resuspended in 20 µl $H_2O$. 20 ng of this vector was ligated with 20 ng of the 39 bp fragment prepared above for 4 hours at room temperature. One-fifth of the ligation mix was used to transform *E. coli* strain 294 to ampicillin resistance (on LB +20 µg/ml) amp plates. Plasmids from the transformants were examined by a quick screen procedure (44): One plasmid, pPGK-39 was selected for sequence analysis. 20 µg of this plasmid was digested with *Xba*I, ethanol precipitated then treated with 1000 units of bacterial alkaline phosphase at 68°C for 45 min. The DNA was 3× phenol-chloroform extracted, then ethanol precipitated. The dephosphorylated ends were then labelled in a 20 µl reaction containing 200 µCi of [$\gamma^{32}$-P] ATP and 10 units of $T_4$ polynucleotide kinase. The plasmid was cut with *Sal*I and run on a 6 percent acrylamide gel.

The labelled insert band was isolated from the gel and sequenced by the chemical degradation method (52). The DNA sequence at the 3'-end of this promoter piece was as expected.

2. Construction of 304 pb *Pvu*I-to-*Eco*RI PGK promoter fragment

25 µg of pPGK-39 (Figure 13) was simultaneously digested with *Sal*I and *Xba*I (5 units each) then electrophoresed on a 6 percent gel. The 390 bp band containing the 39 bp promoter piece was isolated by electroelution. The resuspended DNA was restricted with *Sau*3A then electrophoresed on an 8 percent acrylamide gel. The 39 bp PGK promoter band was isolated by electroelution. This DNA contained 39 bp of the 5' end of the PGK promoter on a *Sau*3A-to-*Xba*I fragment.

25 µg of pB1 was restricted with *Pvu*I and *Kpn*I then electrophoresed on a 6 percent acrylamide gel. The 0.8 kbp band of DNA was isolated by electroelution, then restricted with *Sau*3A and electrophoresed on a 6 percent acrylamide gel. The 265 bp band from the PGK promoter (Figure 11) was isolated by electroelution.

This DNA was then ligated with the 39 bp promoter fragment from above for two hours at room temperature. The ligation mix was restricted with *Xba*I and *Pvu*I then electrophoresed on a 6 percent acrylamide gel. The 304 bp *Xba*-to-*Pvu*I restriction fragment was isolated by electroelution, then added to a ligation mix containing 200 ng of pBR322 (41) (previously isolated missing the 162 bp *Pvu*I-to-*Pst*I restriction fragment) and 200 ng of the *Xba*I-to-PstI LeIF A cDNA gene previously isolated from 20 µg of pLeIF trp A 25. This three-factor-ligation mix was used to transform *E. coli* strain 294 to tetracycline resistance. Transformant colonies were miniscreened (44) and one of the colonies, pPGK-300 was isolated as having 304 bp of PGK 5'-flanking DNa fused to the LeIF A gene in a pBR322 based vector. The 5' end of the LeIF A gene has the following sequence: 5'-CTAGAATTC-3'. Thus fusion of the *Xba*I site from the PGK promoter fragment into this sequence allows for the addition to the *Xba*I site an *Eco*RI site. pPGK-300 thus contains part of the PGK promoter isolated in a *Pvu*I-to-*Eco*RI fragment.

3. Construction of a 1500 bp *Eco*RI-to-*Eco*RI PGK promoter fragment

10 µg of pB1 was digested with *Pvu*I and *Eco*RI and run on a 6 percent acrylamide gel. The 1.3 kb *Pvu*I-to-*Eco*RI DNA band from the PGK 5'-flanking DNA was isolated by electroelution. 10 µg of pPGK-300 was digested with *Eco*RI and *Pvu*I and the 304 bp promoter fragment was isolated by electroelution after electrophoresing the digestion mix on a 6 percent acrylamide gel. 5 µg of pFRL4 was cut with *Eco*RI, ethanol precipitated then treated with bacterial alkaline phosphatase at 68°C for 45 minutes. After three extractions of DNA with phenol/chloroform, ethanol precipitation, and resuspension in 20 ml of $H_2O$; 200 ng of the vector was ligated with 100 ng of 312 bp *Eco*RI-to-*Pvu*I DNA from pPGK-300 and 100 ng of *Eco*RI-to-*Pvu*I DNA from pB1. The ligation mix was used to transform *E. coli* strain 294 to ampicillin resistance. One of the transformants obtained was pPGK-1500. This plasmid contains the 1600 bp PGK promoter fragment as an *Eco*RI-to-*Eco*RI or *Hin*dIII-to-*Eco*RI piece of DNA.

10 µg of pPGK-1500 was completely digested with *Cla*I and *Eco*RI then the digestion mix was electrophoresed on a 6 percent acrylamide gel. The 900 bp fragment containing the PGK promoter was isolated by electroelution. 10 µg of pIFN-γ trp 48 was completely digested with *Eco*RI and *Hin*cII and electrophoresed on a 6 percent acrylamide gel. The 938 bp band containing the directly expressable IFN-γ cDNA was isolated from the gel by electroelution.

The yeast expression vector was constructed in a three factor reaction by ligating together the PGK promoter fragment (on a *Cla*I-to-*Eco*RI piece), the deleted pFRM-31 and the above isolated IFN-γ cDNA. The ligation reaction was incubated at 14°C for 12 hours. The ligation mix was then used to transform *E. coli* strain 294 to ampicillin resistance. Transformants were analyzed for the presence of the properly constructed expression plasmid pPGK-IFN-γ (Figure 16). Plasmids containing the expression system were used to transform spheroplasts of yeast strain RH218 to tryptophan prototropy in agar missing tryptophan. These recombinant yeast were then assayed for the presence of recombinant human immune interferon.

Yeast extracts were prepared as follows: Ten ml cultures were grown in YNB+CAA until reaching $A_{660}$~1—2, collected by centrifugation then resuspended in 500 µl PBS buffer (20 mM $NaH_2PO_4$, pH=7.4, 150 mM NaCl). An equal volume of glass beads (0.45—0.5 mm) were added and the mixture was then vortexed for 2'. The extracts were spun 30 seconds at 14,000 rpm and supernatant removed. Interferon activity in the supernatant was determined to be 16,000 units/ml by comparison with IFN-α standard using the CPE inhibition assay.

M. Construction of cell culture vector pSVγ69

The 342 base pair *Hin*dIII-*Pvu*II fragment encompassing the SV40 origin was converted to an *Eco*RI restriction site bound fragment. The *Hin*dIII site was converted by the addition of a synthetic oligomer (5'dAGCTGAATTC) and the *Pvu*II site was converted by blunt-end ligation into an *Eco*RI site filled in using Polymerase I (Klenow fragment). The resulting *Eco*RI fragment was inserted into the *Eco*RI site of pML-1 (28). A plasmid with the SV40 late promoter oriented away from the $amp^R$ gene was further modified by removing the *Eco*RI site nearest the $amp^R$ gene of pML-1 (27).

The 1023 base pair *Hpa*I-*Bgl*II fragment of cloned HBV DNA (60) was isolated and the *Hpa*I site of hepatitis B virus (HBV) converted to an *Eco*RI site with a synthetic oligomer (5'dGCGAATTCGC). This *Eco*RI-*Bgl*II bounded fragment was directly cloned into the *Eco*RI-*Bam*HI sites of the plasmid described above carrying the origin of SV40.

Into the remaining *Eco*RI site was inserted the IFN-γ gene on a 1250 base pair *Pst*I fragment of p69 after conversion of the *Pst*I ends to *Eco*RI ends. Clones were isolated in which the SV40 late promoter preceded the structural gene of IFN-γ. The resulting plasmids were then introduced into tissue culture cells (29) using a DEAE-dextran technique (61) modified such that the transfection in the presence of DEAE-dextron was carried out for 8 hours. Cell media was changed every 2—3 days. 200 microliters was removed daily for interferon bioassay. Typical yields were 50—100 units/ml on samples assayed three or four days after transfection.

N. Partial purification of monkey cell derived immune interferon

In order to produce greater quantities of the monkey cell derived human IFN-α, fresh monolayers of COS-7 cells in ten 10 cm plates were transfected with a total of 30 µg pDLIF3 in 110 mls DEAE-Dextran (200 µg/ml DEAE Dextran 500,000 MW, .05 M Tris pH 7.5, in DMEM). After 16 hrs at 37°, the plates were washed twice with DMEM. 15 mls fresh DMEM supplemented with 10 percent f.b.s., 2 mM glutamine, 50 µ/ml penicillin G, and 50 mg/ml streptomycin was then added to each plate. The media was replaced the following day with serum-free DMEM. Fresh serum-free media was then added every day. The media collected was kept at 4° until either assayed or bound to CPG. The pooled fractions from 3 and 4 day post-transfection samples were found to contain essentially all of the activity.

0.5 g of CPG (controlled pore glass, Electronucleonics, CPG 350, mesh size 120/200) were added to 100 ml of cell supernatant and the mixture stirred for 3 hrs at 4°C. After a short centrifugation in a bench top centrifuge the settled beads were packed into a column and thoroughly washed with 20 mM $NaPO_4$ 1 M NaCl 0.1 percent β-mercaptoethanol pH 7.2. The activity was then eluted with the same buffer containing 30 percent ethyleneglycol followed by further elution with the above buffer containing 50 percent ethyleneglycol. Basically all the activity bound to the CPG. 75 percent of the eluted activity was found in the fractions eluted with 30 percent ethyleneglycol. These fractions were pooled and diluted with 20 mM $NaPO_4$ 1 M NaCl pH 7.2 to a final concentration of 10 percent ethyleneglycol and directly applied to a 10 ml Con A Sepharose (Pharmacia) column. After a thorough wash with 20 mM $NaPO_4$ 1 M NaCl pH 7.2 the activity was eluted with 20 mM $NaPO_4$ 1 M NaCl 0.2 M α - methyl - D - mannoside. A substantial amount of the activity (55 percent) did not bind to this lectin. 45 percent of the activity eluted with α - methyl - D - mannoside.

Pharmaceutical compositions

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the human immune interferon product hereof is combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are described in Remington's *Pharmaceutical Sciences* by E. W. Martin, which is hereby incorporated by

reference. Such compositions will contain an effective amount of the interferon protein hereof together with a suitable amount of vehicle in order to prepare pharmaceutically acceptable compositions suitable for effective administration to the host.

A. Parenteral administration

The human immune interferon hereof may be parenterally administered to subjects requiring antitumor, or antiviral treatment, and to those exhibiting immunosuppressive conditions. Dosage and dose rate may parallel that currently in use in clinical investigations of other human interferons, e.g., about $(1—10)\times10^6$ units daily, and in the case of materials of purity greater than 1 percent, likely up to, e.g., $50\times10^6$ units daily. Dosages of IFN-γ could be significantly elevated for greater effect owing to the essential absence of human proteins other than IIN-γ, which proteins in human derived materials may induce certain untoward effects.

As one example of an appropriate dosage form for essentially homogeneous IFN-γ in parenteral form applicable herein, 3 mg IFN-γ of specific activity of, say, $2\times10^8$ U/mg may be dissolved in 25 ml. 5 N serum albumin (human)—USP, the solution passed through a bacteriological filter and the filtered solution aseptically subdivided into 100 vials, each containing $6\times10^6$ units pure interferon suitable for parenteral administration. The vials are preferably stored in the cold (−20°C) prior to use.

Bioassay data

A. Characterization of antiviral activity

For antibody neutralizations, samples were diluted, if necessary, to a concentration of 500—1000 units/ml with PBS-BSA. Equal volumes of sample were incubated for 2—12 hrs at 4 degrees with serial dilutions of rabbit antihuman leukocyte, fibroblast, or immune interferon antisera. The anti-IFN-α and β were obtained from the National Institute of Allergy and Infectious Diseases. The anti-IFN-γ was prepared using authentic IFN-γ (5—20 percent purity) purified from stimulated peripheral blood lymphocytes. Samples were centrifuged 3 minutes at 12,000×g for 3 min before assay. To test pH 2 stability, samples were adjusted to pH 2 by addition of 1 N HCl, incubated for 2—12 hrs at 4°, and neutralized by addition of 1 N NaOH before assay. To test sodium dodecyl sulfate (SDS) sensitivity, samples were incubated with an equal volume of 0.2 percent SDS for 2—12 hrs at 4° before assay.

B. Characterization of IFN-γ produced by *E. coli* and COS-7 cells

| | Antiviral activity (units/ml) | | | | |
|---|---|---|---|---|---|
| Treatment | IFN-α | IFN-β | IFN-γ | *E. coli* W3110/ pIFN-γtrp48 extract | COS-7 cell/ pSVγ69 supernatant |
| Untreated | 375 | 125 | 250 | 250 | 62.5 |
| pH 2 | 375 | 125 | <6 | <12 | <4 |
| 0.1 percent SDS | 375 | — | <4 | <8 | — |
| Rabbit anti-IFN-α | <8 | 125 | 250 | 250 | 187 |
| Rabbit anti-IFN-β | 375 | <8 | 187 | 250 | 125 |
| Rabbit anti-IFN-γ | 375 | 125 | <4 | <8 | <4 |

This table shows the characteristic behavior of IFN-α, β and γ standards after various treatments. The interferon activity produced by *E. coli* W3110/pIFN-γ trp 48 and by COS-7/pSVγ69 is acid-sensitive, SDS-sensitive, and neutralized by immune interferon antiserum. It is not neutralized by antibodies to IFN-α or β. These data confirm that the products produced in these systems are immune interferons and that the cDNA insert of plasmid p69 codes for IFN-γ.

Purification

One method by which IFN-γ can be purified from e.g. bacteria is described by the following general scheme:

1. Extraction of the cells in high conductivity lysis buffer (at about pH 8) by passage through a homogenizer at high pressure, cooling the effluent in an ice bath.
2. Precipitation of DNA by polyethylene-imine addition under stirring, for example at 4°C.
3. pH precipitation of bacterial proteins, again leaving IFN-γ in solution.
4. Separation of the solids by centrifugation at 4°C.
5. Concentration of the supernatant (after readjusting the pH) as by ultrafiltration.

6. Dialysis of the concentrate against a low conductivity buffer.

7. Removing solids by centrifugation leaving the IFN-γ in solution.

8. Ion exchange chromatography on carboxymethyl cellulose, eluting with a gradient of increasing ionic strength.

9. Chromatography on calcium phosphate gel by eluting with a gradient of increasing ionic strength.

10. Ion exchange chromatography on carboxymethyl cellulose under weak denaturing conditions by eluting with a gradient of increasing ionic strength.

11. Separation by gel filtration chromatography.

The above process enables yields of material of greater than 95% purity.

The immune interferon protein hereof has been defined by means of determined DNA gene and deductive amino acid sequencing—cf. Figure 5. It will be understood that for this particular interferon, embraced herein, natural allelic variations exist and occur from individual to individual. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. All such allelic variations are included within the scope of this invention.

Indeed, the potential exists in the use of recombinant DNA technology for the preparation of various human IFN-γ derivatives, variously modified by resultant single or multiple amino acid substitutions, deletions, additions or replacements. All such modifications resulting in such derivatives of human IFN-γ are included within the ambit of this invention so long as the essential, characteristic human IFN-γ activity remains unaffected in kind.

With the DNA and amino acid sequences of IFN-γ in hand (see Figure 5), the most preferable course in reproducing the present invention doubtless would involve the preparation of either the complete gene by synthesis means (see 26, for example), or synthetic deoxyoligonucleotides with which the cDNA source could be probed in order to isolate the gene by standard hybridization techniques. Once having obtained the nucleotide sequence encoding the requisite IFN-γ protein, the means for achieving expression, isolation and purification to afford highly pure preparations of IFN-γ could be followed according to the above description.

Notwithstanding that reference has been made to particular preferred embodiments, it will be further understood that the present invention is not to be construed as limited to such, rather to the lawful scope of the appended claims.

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**Claims for the Contracting States: BE, CH, DE, FR, GB, IT, LI, LU, NL, SE**

1. Human immune interferon of the amino acid sequence depicted in Figure 5 hereof and alleles thereof, free from other protein with which it is ordinarily associated.

2. A derivative of human immune interferon of the amino acid sequence depicted in Figure 5 hereof and alleles thereof, said derivative having the function of human immune interferon.

3. A human immune interferon derivative according to claim 2 having the amino acid methionine at the N-terminus.

4. A human immune interferon according to claim 3 having the amino acid methionine at the N-terminus of the mature sequence.

5. Human immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative thereof having the function of human immune interferon, unaccompanied by associated native glycosylation.

6. Human immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative thereof having the function of human immune interferon, as produced by the expression in a recombinant host cell of DNA encoding the amino acid sequence of the interferon.

7. Human immune interferon of claim 6, as produced by expression of a recombinant DNA sequence coding therefor in a mammalian cell line.

8. Human immune interferon of claim 7 wherein the cell line is a COS-7 monkey cell line.

9. A DNA isolate comprising a DNA sequence encoding human immune interferon having the amino acid sequence depicted in Figure 5 hereof or an allele or derivative thereof having the function of human immune interferon.

10. A recombinant cloning vector comprising a DNA sequence encoding human immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative having the function of human immune interferon.

11. A replicable expression vector capable, in a transformant microorganism or cell culture, of expressing a DNA sequence encoding human immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative having the function of human immune interferon.

12. A recombinant microorganism or cell culture capable of expressing immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative thereof having the function of human immune interferon.

13. A microorganism according to claim 12, which is an *E. coli* strain.

14. A microorganism according to claim 12 which is a yeast strain.

15. A cell culture according to claim 12, which is a mammalian cell line.

16. A cell culture according to claim 15 wherein the cell line is a COS-7 monkey cell line.

17. A composition comprising a therapeutically effective amount of human immune interferon according to any one of claims 1 to 8 in admixture with a pharmaceutically acceptable carrier.

18. The composition according to claim 17 suitable for parenteral administration.

19. A process which comprises expressing human immune interferon in a mammalian cell line transformed with the DNA depicted in Figure 5 hereof or an allelic variant thereof.

20. A process according to claim 19 wherein the cell line is a COS-7 monkey cell line.

21. A process according to claim 19 or claim 20 which includes recovering the human immune interferon from the culture.

22. A process which comprises expressing in a recombinant host cell DNA encoding human immune interferon as obtainable by the process of claim 21.

23. A process which comprises expressing in a recombinant host cell DNA encoding human immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative thereof having the function of human immune interferon.

24. A process of claim 22 or claim 23, additionally comprising the step of recovering said human immune interferon.

25. A process for producing human immune interferon, which process comprises:

(a) culturing recombinant host cells which have been transformed with an expression vector containing DNA encoding human immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative thereof having the function of human immune interferon under conditions permitting expression of said DNA sequence to produce said human immune interferon; and recovering said human immune interferon.

26. A process according to claim 25, which includes the preliminary steps of

(i) preparing a replicable expression vector capable of expressing a DNA sequence encoding said human immune interferon in a host cell; and

(ii) transforming a host cell culture to obtain said recombinant host cells.

27. A process according to any one of claims 22 to 26 wherein the host is an *E. coli* strain.

28. A process according to any one of claims 22 to 26 wherein the host is a yeast strain.

29. A process according to any one of claims 22 to 26 wherein the host cell is a mammalian cell line.

30. A process according to claim 29 wherein the cell line is a COS-7 monkey cell line.

31. The use of human immune interferon according to any one of claims 1 to 8, or as prepared by a process of any one of claims 19 to 30, in preparing pharmaceutical compositions.

**Claims for the Contracting State: AT**

1. A method which comprises the preparation of human immune interferon of the amino acid sequence depicted in Figure 5 hereof and alleles thereof, free from other protein with which it is ordinarily associated.

2. A method which comprises the preparation of a derivative of human immune interferon of the amino acid sequence depicted in Figure 5 hereof and alleles thereof, said derivative having the function of human immune interferon.

3. A method according to claim 2 wherein the derivative has the amino acid methionine at the N-terminus.

4. A method according to claim 3 wherein the derivative has the amino acid methionine at the N-terminus of the mature sequence.

5. A method which comprises the preparation of human immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative thereof having the function of human immune interferon, unaccompanied by associated native glycosylation.

6. A method which comprises the preparation of a DNA isolate comprising a DNA sequence encoding human immune interferon having the amino acid sequence depicted in Figure 5 hereof or an allele or derivative thereof having the function of human immune interferon.

7. A method which comprises the preparation of a recombinant cloning vector comprising a DNA sequence encoding human immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative having the function of human immune interferon.

8. A method which comprises the preparation of a replicable expression vector capable, in a transformant microorganism or cell culture, of expressing a DNA sequence encoding human immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative having the function of human immune interferon.

9. A recombinant microorganism or cell culture capable of expressing immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative thereof having the function of human immune interferon.

10. A microorganism according to claim 9, which is an *E. coli* strain.

11. A microorganism according to claim 9 which is a yeast strain.

12. A cell culture according to claim 9 which is a mammalian cell line.

13. A cell culture according to claim 12 wherein the cell line is a COS-7 monkey cell line.

14. A process which comprises expressing human immune interferon in a mammalian cell line transformed with the DNA depicted in Figure 5 hereof or an allelic variant thereof.

15. A process according to claim 14 wherein the cell line is a COS-7 monkey cell line.

16. A process according to claim 14 or claim 15 which includes recovering the human immune interferon from the culture.

17. A process which comprises expressing in a recombinant host cell DNA encoding human immune interferon as obtainable by the process of claim 16.

18. A process which comprises expressing in a recombinant host cell DNA encoding human immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative thereof having the function of human immune interferon.

19. A process of claim 17 or claim 18, additionally comprising the step of recovering said human immune interferon.

20. A process for producing human immune interferon, which process comprises:

(a) culturing recombinant host cells which have been transformed with an expression vector containing DNA encoding human immune interferon of the amino acid sequence depicted in Figure 5 hereof or an allele or derivative thereof having the function of human immune interferon under conditions permitting expression of said DNA sequence to produce said human immune interferon; and recovering said human immune interferon.

21. A process according to claim 20, which includes the preliminary steps of

(i) preparing a replicable expression vector capable of expressing a DNA sequence encoding said human immune interferon in a host cell; and

(ii) transforming a host cell culture to obtain said recombinant host cells.

22. A process according to any one of claims 17 to 21 wherein the host is an *E. coli* strain.

23. A process according to any one of claims 17 to 21 wherein the host is a yeast strain.

24. A process according to any one of claims 17 to 21 wherein the host cell is a mammalian cell line.

25. A process according to claim 24 wherein the cell line is a COS-7 monkey cell line.

26. The use of human immune interferon as produced by the method of any one of claims 1 to 5 and 14 to 25 in preparing pharmaceutical compositions.

**Patentansprüche für die Vertragsstaaten: BE, CH, DE, FR, GB, IT, LI, LU, NL, SE**

1. Human-Immuninterferon mit der vorstehend in Fig. 5 dargestellten Aminosäuresequenz, das frei ist von anderem Protein, mit welchem es gewöhnlich assoziiert ist, sowie Allele davon.

2. Ein Derivat von Human-Immuninterferon mit der vorstehend in Fig. 5 dargestellten Aminosäuresequenz, sowie Allele davon, wobei das genannte Derivat die Funktion von Human-Immuninterferon hat.

3. Ein Human-Immuninterferon-Derivat gemäß Anspruch 2, mit der Aminosäure Methionin am N-Ende.

4. Ein Human-Immuninterferon gemäß Anspruch 3 mit der Aminosäure Methionin am N-Ende der reifen Sequenz.

5. Human-Immuninterferon mit der vorstehend in Fig. 5 dargestellten Aminosäuresequenz, oder ein Allel oder ein Derivat davon mit der Funktion eines Human-Immuninterferons, unbegleitet durch damit assoziierte natürlich Glycosylierung.

6. Human-Immuninterferon mit der vorstehend in Fig. 5 dargestellten Aminosäuresequenz, oder ein Allel oder Derivat davon mit der Funktion eines Humanimmuninterferons, gebildet durch Expression einer DNA, welche für die Aminosäuresequenz von Interferon kodiert, in einer rekombinanten Wirtszelle.

7. Human-Immuninterferon nach Anspruch 6, gebildet durch Expression einer dafür kodierenden rekombinanten DNA Sequenz in einer Zellinie von Säugetieren.

8. Human-Immuninterferon nach Anspruch 6, worin die Zellinie eine COS-7 Affenzellinie ist.

9. Ein DNA-Isolat umfassend eine DNA-Sequenz, welche für Human-Immuninterferon kodiert, das die vorstehend in Fig. 5 dargestellte Aminosäuresequenz aufweist, oder ein Allel oder Derivat davon mit der Funktion von Human-Immuninterferon.

10. Ein rekombinanter Klonierungsvektor beinhaltend eine DNA-Sequenz, die für Human-Immuninterferon kodiert, mit der vorstehend in Fig. 5 dargestellten Aminosäuresequenz, oder ein Allel oder Derivat davon mit der Funktion von Human-Immuninterferon.

11. Ein replizierbarer Expressionsvektor der fähig ist, in einem transformanten Mikroorganismus oder Zellkultur eine DNA-Sequenz, die für Human-Immuninterferon einer vorstehend in Fig. 5 dargestellten Aminosäuresequenz kodiert, oder ein Allel oder Derivat davon mit der Funktion von Human-Immuninterferon zu exprimieren.

12. Ein rekombinanter Mikroorganismus oder Zellkultur, der bzw. die fähig ist, Immuninterferon einer vorstehend in Fig. 5 dargestellten Aminosäuresequenz oder ein Allel oder Derivat davon mit der Funktion von Human-Immuninterferon zu exprimieren.

13. Ein Mikroorganismus nach Anspruch 12, welcher ein *E. coli* Stamm ist.

14. Ein Mikroorganismus nach Anspruch 12, welcher ein Hefe-Stamm ist.

15. Eine Zellkultur nach Anspruch 12, welche eine Säugetierzellinie ist.

16. Eine Zellkultur nach Anspruch 15, worin die Zellinie eine COS-7 Affenzellinie ist.

17. Eine Zusammensetzung beinhaltend eine therapeutisch wirksame Menge von Human-Immuninterferon gemäß irgendeinem der Ansprüche 1 bis 8 in Mischung mit einem pharmazeutisch verträglichen Träger.

18. Die Zusammensetzung gemäß Anspruch 17 geeignet für parenterale Verabreichung.

19. Ein Verfahren, welches die Expression von Human-Immuninterferon in einer Säugetierzellinie, die mit der vorstehend in Fig. 5 dargestellten DNA transformiert ist, oder einer allelischen Variante davon umfaßt.

20. Ein Verfahren nach Anspruch 19, worin die Zellinie eine COS-7 Affenzellinie ist.

21. Ein Verfahren nach Anspruch 19 oder Anspruch 20, welches die Gewinnung von Human-Immuninterferon aus der Kultur einschließt.

22. Ein Verfahren, welches die Expression, einer DNA, die für Human-Immuninterferon, das nach dem Verfahren nach Anspruch 21 erhältlich ist, kodiert, in einer rekombinanten Wirtszelle umfaßt.

23. Ein Verfahren, welches die Expression einer für Human-Immuninterferon der vorstehend in Fig. 5 angegebenen Aminosäuresequenz oder ein Allel oder Derivat davon, die die Funktion von Human-Immuninterferon besitzen, kodierenden DNA in einer rekombinanten Wirtszelle umfaßt.

24. Ein Verfahren nach Anspruch 22 oder Anspruch 23, welches zusätzlich die Stufe die Gewinnung des genannten Human-Immuninterferons umfaßt.

25. Ein Verfahren zur Herstellung von Human-Immuninterferon, welches Verfahren

a) die Züchtung rekombinanter Wirtszellen, die mit einem Expressionsvektor transformiert werden, der eine DNA enthält, die für Human-Immuninterferon der vorstehend in Fig. 5 dargestellten Aminosäuresequenz oder ein Allel oder Derivat davon, die die Funktion von Human-Immuninterferon aufweisen, kodiert, unter Bedingungen, die die Expression der genannten DNA-Sequenz zulassen, um das genannte Human-Immuninterferon herzustellen, sowie die Gewinnung des genannten Human-Immuninterferons umfaßt.

26. Ein Verfahren nach Anspruch 25, welches die vorbereitenden Schritte der

(i) Herstellung eines replizierbaren Expressionsvektors, der fähig ist, eine DNA-Sequenz, die für das genannte Human-Immuninterferon kodiert, in einer Wirtszelle zu exprimieren, und

(ii) Transformation einer Wirtszellenkultur, um die erwähnten rekombinanten Wirtszellen zu erhalten, umfaßt.

27. Ein Verfahren nach einem der Ansprüche 22 bis 26, worin der Wirt ein *E. coli* Stamm ist.

28. Ein Verfahren nach einem der Ansprüche 22 bis 26, worin der Wirt eine Hefe-Stamm ist.

29. Ein Verfahren nach einem der Ansprüche 22 bis 26, worin die Wirtszelle eine Säugetierzellinie ist.

30. Ein Verfahren nach Anspruch 29, worin die Zellinie eine COS-7 Affenzellinie ist.

31. Die Verwendung von Human-Immuninterferon nach einem der Ansprüche 1 bis 8, oder wie nach einem Verfahren nach einem der Ansprüche 19 bis 30 hergestellt, bei der Herstellung von pharmazeutischen Zusammensetzungen.

**Patentansprüche für den Vertragsstaat: AT**

1. Verfahren umfassen die Herstellung von Human-Immuninterferon mit der vorstehend in Fig. 5 dargestellten Aminosäuresequenz, das frei ist von anderem Protein, mit welchem es gewöhnlich assoziiert ist, sowie von Allelen davon.

2. Verfahren umfassend die Herstellung eines Derivats von Human-Immuninterferon mit der vorstehend in Fig. 5 dargestellten Aminosäuresequenz sowie von Allelen davon, wobei das genannte Derivat die Funktion von Human-Immuninterferon hat.

3. Verfahren nach Anspruch 2, worin das Derivat die Aminosäure Methionin am N-Ende aufweist.

4. Verfahren nach Anspruch 3, worin das Derivat die Aminosäure Methionin am N-Ende der reifen Sequenz aufweist.

5. Verfahren umfassend die Herstellung von Human-Immuninterferon mit der vorstehend in Fig. 5 dargestellten Aminosäuresequenz oder eines Allels oder Derivats davon mit der Funktion eines Human-Immuninterferons, unbegleitet durch damit assoziierte natürliche Glycosylierung.

6. Verfahren umfassend die Herstellung eines DNA-Isolats umfassend eine DNA-Sequenz, welche für Human-Immuninterferon kodiert, das die vorstehend in Fig. 5 dargestellte Aminosäuresequenz aufweist, oder eines Allels oder Derivats davon mit der Funktion von Human-Immuninterferon.

7. Verfahren umfassend die Herstellung eines rekombinanten Klonierungsvektors beinhaltend eine DNA-Sequenz, die für Human-Immuninterferon kodiert, mit der vorstehend in Fig. 5 dargestellten Aminosäuresequenz, oder eines Allels oder Derivats davon mit der Funktion von Human-Immuninterferon.

8. Verfahren umfassend die Herstellung eines replizierbaren Expressionsvektors, der fähig ist, in einem transformanten Mikroorganismus oder Zellkultur eine DNA-Sequenz, die für Human-Immuninterferon einer vorstehend in Fig. 5 dargestellten Aminosäuresequenz kodiert, oder eines Allels oder Derivats davon mit der Funktion von Human-Immuninterferon zu exprimieren.

9. Ein rekombinanter Mikroorganismus oder Zellkultur, der bzw. die fähig ist, Immuninterferon einer

vorstehend in Fig. 5 dargestellten Aminosäuresequenz oder eines Allels oder Derivats davon mit der Funktion von Human-Immuninterferon zu exprimieren.

10. Ein Mikroorganismus nach Anspruch 9, welcher ein *E. coli* Stamm ist.

11. Ein Mikroorganismus nach Anspruch 9, welcher ein Hefe-Stamm ist.

12. Eine Zellkultur nach Anspruch 9, welche eine Säugetierzellinie ist.

13. Eine Zellkultur nach Anspruch 12, worin die Zellinie eine COS-7 Affenzellinie ist.

14. Verfahren, welches die Expression von Human-Immuninterferon in einer Säugetierzellinie, die mit der vorstehend in Fig. 5 dargestellten DNA transformiert ist, oder einer allelischen Variante davon umfaßt.

15. Verfahren nach Anspruch 14, worin die Zellinie eine COS-7 Affenzellinie ist.

16. Verfahren nach Anspruch 14 oder Anspruch 15, welches die Gewinnung von Human-Immuninterferon aus der Kultur einschließt.

17. Verfahren, welches die Expression einer DNA, die für Human-Immuninterferon, das nach dem Verfahren nach Anspruch 16 erhältlich ist, kodiert, in einer rekombinanten Wirtszelle umfaßt.

18. Verfahren, welches die Expression einer für Human-Immuninterferon der vorstehend in Fig. 5 angegebenen Aminosäuresequenz oder eine Allel oder Derivat davon, die die Funktion von Human-Immuninterferon besitzen, kodierenden DNA in einer rekombinanten Wirtszelle umfaßt.

19. Verfahren nach Anspruch 17 oder Anspruch 18, welches zusätzlich die Stufe der Gewinnung des genannten Human-Immuninterferons umfaßt.

20. Verfahren zur Herstellung von Human-Immuninterferon, welches Verfahren

a) die Züchtung rekombinanter Wirtszellen, die mit einem Expressionsvektor transformiert werden, der eine DNA enthält, die für Human-Immuninterferon der vorstehend in Fig. 5 dargestellten Aminosäuresequenz oder ein Allel oder Derivat davon, die die Funktion von Human-Immuninterferon aufweisen, kodiert, unter Bedingungen, die die Expression der genannten DNA-Sequenz zulassen, um das genannte Human-Immuninterferon herzustellen, sowie die Gewinnung des genannten Human-Immuninterferons umfaßt.

21. Verfahren nach Anspruch 20, welches die vorbereitenden Schritte der

(i) Herstellung eines replizierbaren Expressionsvektors, der fähig ist, eine DNA-Sequenz, die für das genannte Human-Immuninterferon kodiert, in einer Wirtszelle zu exprimieren, und

(ii) Transformation einer Wirtszellenkultur, um die erwähnten rekombinanten Wirtszellen zu erhalten, umfaßt.

22. Verfahren nach einem der Ansprüche 17 bis 21, worin der Wirt ein *E. coli* Stamm ist.

23. Verfahren nach einem der Ansprüche 17 bis 21, worin der Wirt ein Hefe-Stamm ist.

24. Verfahren nach einem der Ansprüche 17 bis 21, worin die Wirtszelle eine Säugetierzellinie ist.

25. Verfahren nach Anspruch 24, worin die Zellinie eine COS-7 Affenzellinie ist.

26. Die Verwendung von Human-Immuninterferon, hergestellt nach dem Verfahren nach einem der Ansprüche 1 bis 5 und 14 bis 25, bei der Herstellung von pharmazeutischen Zusammensetzungen.

**Revendications pour les Etats Contractants: BE, CH, DE, FR, GB, IT, LI, LU, NL, SE**

1. Interféron immun humain de la séquence d'acides aminés représentée à la figure 5 et ses allèles, exempt d'autre protéine avec laquelle il est ordinairement associé.

2. Dérivé d'interféron immun humain de la séquence d'acides aminés représentée à la figure 5 et ses allèles, ledit dérivé ayant la fonction de l'interféron immun humain.

3. Dérivé d'interféron immun humain selon la revendication 2 ayant l'acide aminé méthionine à l'extrémité N.

4. Interféron immun humain selon la revendication 3 ayant l'acide aminé méthionine à l'extrémité N de la séquence mûre.

5. Interféron immun humain de la séquence d'acides aminés représentée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain sans être accompagné d'une glycosylation naturelle associée.

6. Interféron immun humain de la séquence d'acides aminés représentée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain, produit par l'expression dans une cellule hôte recombinante d'un ADN codant la séquence d'acides aminés de l'interféron.

7. Interféron immun humain de la revendication 6 produit par l'expression d'une séquence d'ADN recombinant codant pour lui dans une lignée de cellules de mammifère.

8. Interféron immun humain de la revendication 7 où la lignée de cellules est une lignée de cellules de singe COS-7.

9. Isolat d'ADN comprenant une séquence d'ADN codant l'interféron immun humain ayant la séquence d'acides aminés représentée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain.

10. Vecteur clonant recombinant comprenant une séquence d'ADN codant l'interféron immun humain de la séquence d'acides aminés représentée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain.

11. Vecteur d'expression réplicable capable, dans un micro-organisme transformant ou une culture de

cellules, d'exprimer une séquence d'ADN codant l'interféron immun humain de la séquence d'acides aminés représentée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain.

12. Micro-organisme recombinant ou culture de cellules capable d'exprimer l'interféron immun humain de la séquence d'acides aminés représentée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain.

13. Micro-organisme selon la revendication 12 qui est une souche de *E. coli*.

14. Micro-organisme selon la revendication 12 qui est une souche de levure.

15. Culture de cellules selon la revendication 12, qui est une lignée de cellules de mammifère.

16. Culture de cellules selon la revendication 15 où la lignée de cellules est une lignée de cellules de singe COS-7.

17. Composition comprenant une quantité thérapeutiquement efficace d'interféron immun humain selon l'une quelconque des revendications 1 à 8 en mélange avec un véhicule acceptable en pharmacie.

18. Composition selon la revendication 17 appropriée à une administration parentérale.

19. Procédé qui comprend l'expression de l'interféron immun humain dans une lignée de cellules de mammifère transformée par l'ADN représenté à la figure 5 ou sa variente allélomorphe.

20. Procédé selon la revendication 19 où la lignée de cellules est une lignée de cellules de singe COS-7.

21. Procédé selon la revendication 19 ou la revendication 20 qui comprend la récupération de l'interféron immun humain de la culture.

22. Procédé qui comprend l'expression, dans une cellule hôte recombinante, d'un ADN codant l'interféron immun humain pouvant être obtenu par le procédé de la revendication 21.

23. Procédé qui comprend l'expression, dans une cellule hôte recombinante d'un ADN codant l'interféron immun humain de la séquence d'acides aminés représentée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain.

24. Procédé de la revendication 22 ou la revendication 23 comprenant additionnellement l'étape de récupération dudit interféron immun humain.

25. Procédé de production de l'interféron immun humain, lequel comprend:

(a) la mise en culture de cellules hôtes recombinantes qui ont été transformées par un vecteur d'expression contenant un ADN codant l'interféron immun humain de la séquence d'acides aminés montrée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain dans des conditions permettant l'expression de ladite séquence d'ADN pour produire ledit interféron immun humain; et la récupération dudit interféron immun humain.

26. Procédé selon la revendication 25, qui comprend les étapes préliminaires de

(i) préparer un vecteur d'expression réplicable capable d'exprimer une séquence d'ADN codant ledit interféron immun humain dans une cellule hôte; et

(ii) transformer une culture de cellules hôtes pour obtenir lesdites cellules hôtes recombinantes.

27. Procédé selon l'une quelconque des revendications 22 à 26 où l'hôte est une souche de *E. coli*.

28. Procédé selon l'une quelconque des revendications 22 à 26 où l'hôte est une souche de levure.

29. Procédé selon l'une quelconque des revendications 22 à 26 où la cellule hôte est une lignée de cellules de mammifère.

30. Procédé selon la revendication 29 où la lignée de cellules est une lignée de cellules de singe COS-7.

31. Utilisation de l'interféron immun humain selon l'une quelconque des revendications 1 à 8, ou tel que préparé par un procédé selon l'une quelconque des revendications 19 à 30, pour la préparation de compositions pharmaceutiques.

**Revendications pour l'Etat Contractant: AT**

1. Méthode qui comprend la préparation d'interféron immun humain de la séquence d'acides aminés montrée à la figure 5 et ses allèles, sans autre protéine avec laquelle il est ordinairement associé.

2. Méthode qui comprend la préparation d'un dérivé de l'interféron immun humain de la séquence d'acides aminés représentée à la figure 5 et ses allèles, ledit dérivé ayant la fonction de l'interféron immun humain.

3. Méthode selon la revendication 2 où le dérivé a l'acide aminé méthionine à l'extrémité N.

4. Méthode selon la revendication 3 où le dérivé a l'acide aminé méthionine à l'extrémité N de la séquence mûre.

5. Méthode qui comprend la préparation d'interféron immun humain de la séquence d'acides aminés montrée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain sans accompagnement d'une glycosylation naturelle associée.

6. Méthode qui comprend la préparation d'un isolat d'ADN comprenant une séquence d'ADN codant l'interféron immun humain ayant la séquence d'acides aminés représentée à la figure 5 ou son allèle ou dérivé ayant la fonction d'un interféron immun humain.

7. Méthode qui comprend la préparation d'un vecteur clonant recombinant comprenant une séquence d'ADN codant l'interféron immun humain de la séquence d'acides aminés représentée à la figure 5 ou un allèle ou dérivé ayant la fonction de l'interféron immun humain.

8. Méthode qui comprend la préparation d'un vecteur d'expression réplicable capable, dans un micro-organisme transformant ou une culture de cellules, d'exprimer une séquence d'ADN codant

l'interféron immun humain de la séquence d'acides aminés montrée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain.

9. Micro-organisme recombinant ou culture de cellules capable d'exprimer l'interféron immun de la séquence d'acides aminés représentée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain.

10. Micro-organisme selon la revendication 9, qui est une souche de *E. coli*.

11. Micro-organisme selon la revendication 9 qui est une souche de levure.

12. Culture de cellules selon la revendication 9 qui est une lignée de cellules de mammifère.

13. Culture de cellules selon la revendication 12 où la lignée de cellules est une lignée de cellules de singe COS-7.

14. Procédé qui comprend l'expression de l'interféron immun humain dans une lignée de cellules de mammifère transformée par l'ADN représenté à la figure 5 ou sa variante allélomorphe.

15. Procédé selon la revendication 14 où la lignée de cellules est une lignée de cellules de singe COS-7.

16. Procédé selon la revendication 14 ou la revendication 15 qui comprend la récupération de l'interféron immun humain de la culture.

17. Procédé qui comprend l'expression dans une cellule hôte recombinante d'un ADN codant l'interféron immun humain obtenu par le procédé de la revendication 16.

18. Procédé qui comprend l'expression dans une cellule hôte recombinante d'un ADN codant l'interféron immun humain de la séquence d'acides aminés représentée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain.

19. Procédé selon la revendication 17 ou la revendication 18 comprenant additionnellement l'étape de récupérer ledit interféron immun humain.

20. Procédé de production de l'interféron immun humain, lequel procédé comprend:

(a) la mise en culture de cellules hôtes recombinantes qui ont été transformées avec un vecteur d'expression contenant un ADN codant l'interféron immun humain de la séquence d'acides aminés représentée à la figure 5 ou son allèle ou dérivé ayant la fonction de l'interféron immun humain dans des conditions permettant l'expression de ladite séquence d'ADN pour produire ledit interféron immun humain; et la récupération dudit interféron immun humain.

21. Procédé selon la revendication 20, qui comprend les étapes préliminaires de

(i) préparer un vecteur d'expression réplicable capable d'exprimer une séquence d'ADN codant ledit interféron immun humain dans une cellule hôte; et

(ii) transformer une culture de cellules hôtes pour obtenir lesdites cellules hôtes recombinantes.

22. Procédé selon l'une quelconque des revendications 17 à 21 où l'hôte est une souche de *E. coli*.

23. Procédé selon l'une quelconque des revendications 17 à 21 où l'hôte est une souche de levure.

24. Procédé selon l'une quelconque des revendications 17 à 21 où la cellule hôte est une lignée de cellules de mammifère.

25. Procédé selon la revendication 24 où la lignée de cellules est une lignée de cellules de singe COS-7.

26. Utilisation de l'interféron immun humain tel que produit par la méthode selon l'une quelconque des revendications 1 à 5 et 14 à 25 pour préparer des compositions pharmaceutiques.

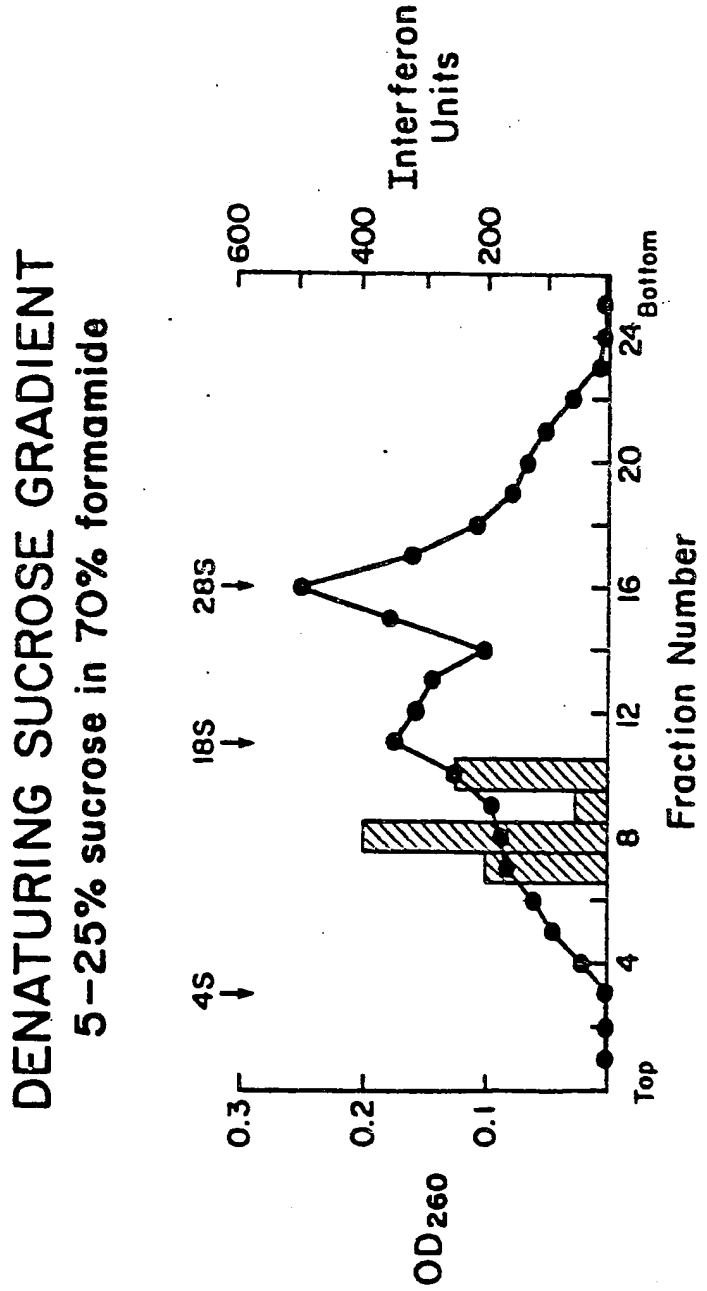

DENATURING SUCROSE GRADIENT
5–25% sucrose in 70% formamide
Interferon Units
600
400
200
OD260
0.3
0.2
0.1
4S
18S
28S
Top
4
8
12
16
20
24
Bottom
Fraction Number

FIG. 1.

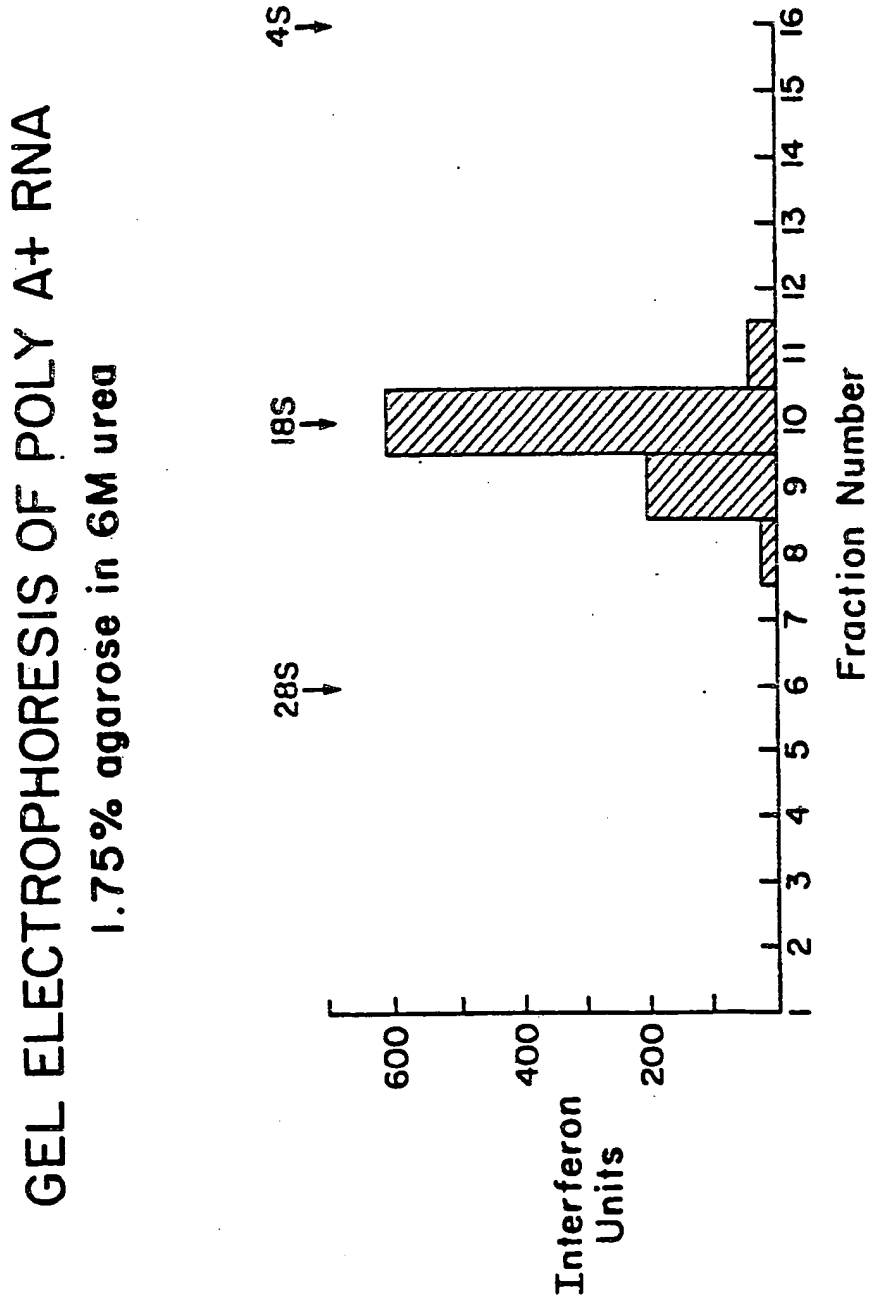
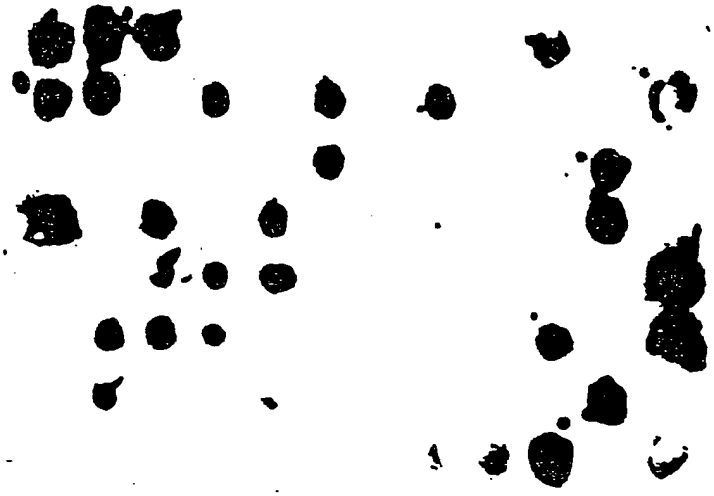

GEL ELECTROPHORESIS OF POLY A+ RNA
1.75% agarose in 6M urea
4S
18S
28S
600
400
200
Interferon Units
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Fraction Number

FIG. 2.

INDUCED $^{32}$P-cDNA Probe




UNINDUCED $^{32}$P-cDNA Probe

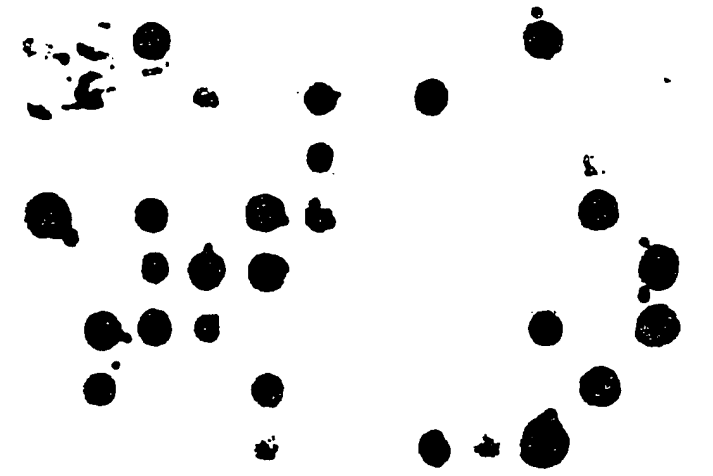



FIG. 3.

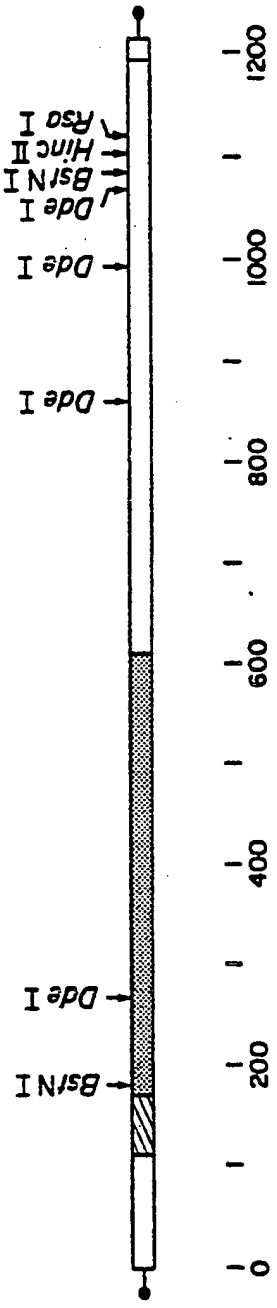

Rsa I
Hinc II
BsiN I
Dde I
Dde I
Dde I
Dde I
BsiN I
0
200
400
600
800
1000
1200

FIG. 4.

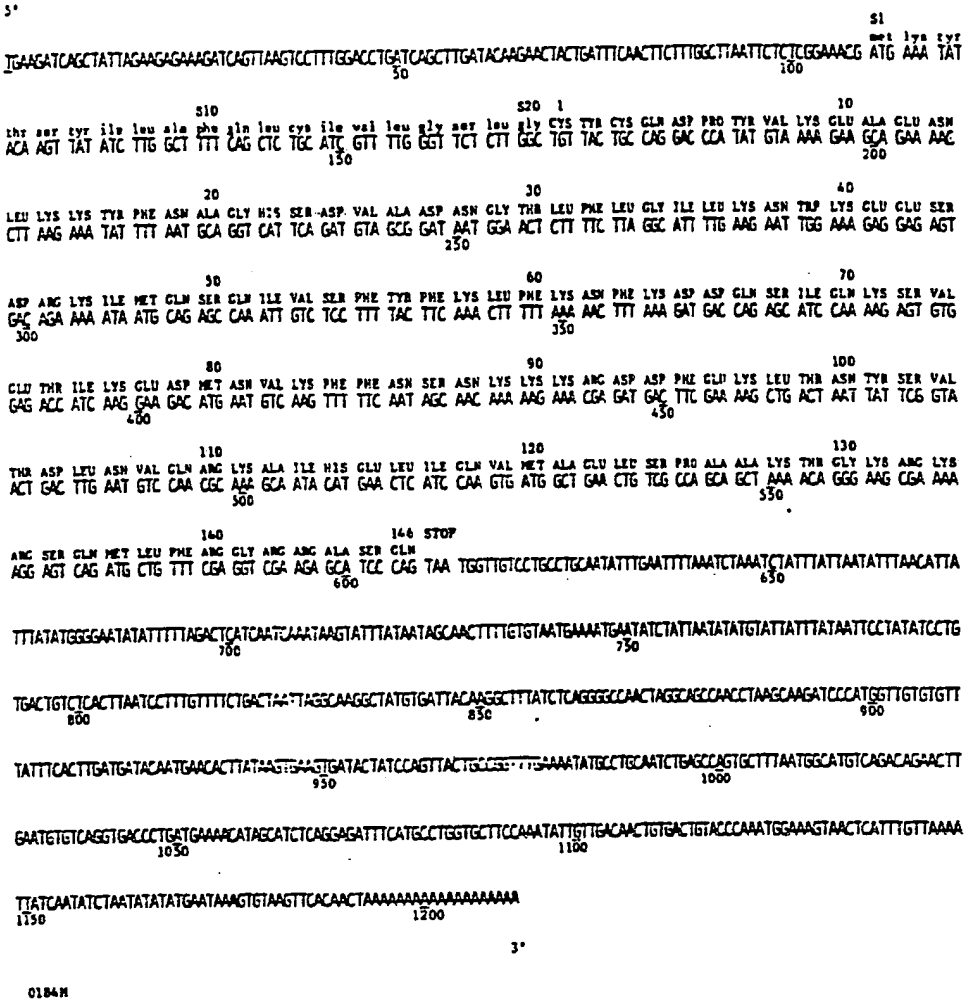

5'
-51
met lys tyr
TGAAGATCAGCTATTAGAAGAGAAAGATCAGTTAAGTCCTTTGGACCTGATCAGCTTGATACAAGAACTACTGATTTCAACTTCTTTGGCTTAATTCTCTCGGAAACG ATG AAA TAT
-10 1 10
thr ser tyr ile leu ala phe gln leu cys ile val leu gly ser leu gly CYS TYR CYS GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN
ACA AGT TAT ATC TTG GCT TTT CAG CTC TGC ATC GTT TTG GGT TCT CTT GGC TGT TAC TGC CAG GAC CCA TAT GTA AAA GAA GCA GAA AAC
20 30 40
LEU LYS LYS TYR PHE ASN ALA GLY HIS SER ASP VAL ALA ASP ASN GLY THR LEU PHE LEU GLY ILE LEU LYS ASN TRP LYS GLU GLU SER
CTT AAG AAA TAT TTT AAT GCA GGT CAT TCA GAT GTA GCG GAT AAT GGA ACT CTT TTC TTA GGC ATT TTG AAG AAT TGG AAA GAG GAG AGT
50 60 70
ASP ARG LYS ILE MET GLN SER GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS ASN PHE LYS ASP ASP GLN SER ILE GLN LYS SER VAL
GAC AGA AAA ATA ATG CAG AGC CAA ATT GTC TCC TTT TAC TTC AAA CTT TTT AAA AAC TTT AAA GAT GAC CAG AGC ATC CAA AAG AGT GTG
80 90 100
GLU THR ILE LYS GLU ASP MET ASN VAL LYS PHE PHE ASN SER ASN LYS LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN TYR SER VAL
GAG ACC ATC AAG GAA GAC ATG AAT GTC AAG TTT TTC AAT AGC AAC AAA AAG AAA CGA GAT GAC TTC GAA AAG CTG ACT AAT TAT TCG GTA
110 120 130
THR ASP LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN VAL MET ALA GLU LEU SER PRO ALA ALA LYS THR GLY LYS ARG LYS
ACT GAC TTG AAT GTC CAA CGC AAA GCA ATA CAT GAA CTC ATC CAA GTG ATG GCT GAA CTG TCG CCA GCA GCT AAA ACA GGG AAG CGA AAA
140 146 STOP
ARG SER GLN MET LEU PHE ARG GLY ARG ARG ALA SER GLN
AGG AGT CAG ATG CTG TTT CGA GGT CGA AGA GCA TCC CAG TAA TGGTTGTCCTGCCTGCAATATTTGAATTTTAAATCTAAATCTATTTATTAATATTTAACATTA
TTTATATGGGGAATATATTTTTAGACTCATCAATCAAATAAGTATTTATAATAGCAACTTTTGTGTAATGAAAATGAATATCTATTAATATATGTATTATTTATAATTCCTATATCCTG
TGACTGTCTCACTTAATCCTTTGTTTTCTGACTAATTAGGCAAGGCTATGTGATTACAAGGCTTTATCTCAGGGGCCAACTAGGCAGCCAACCTAAGCAAGATCCCATGGTTGTGTGTT
TATTTCACTTGATGATACAATGAACACTTATAAGTGAAGTGATACTATCCAGTTACTGCCGCTTTGAAAATATGCCTGCAATCTGAGCCAGTGCTTTAATGGCATGTCAGACAGAACTT
GAATGTGTCAGGTGACCCTGATGAAAACATAGCATCTCAGGAGATTTCATGCCTGGTGCTTCCAAATATTGTTGACAACTGTGACTGTACCCAAATGGAAAGTAACTCATTTGTTAAAA
TTATCAATATCTAATATATATGAATAAAGTGTAAGTTCACAACTAAAAAAAAAAAAAAAAAAA
3'
0184M

FIG. 5.

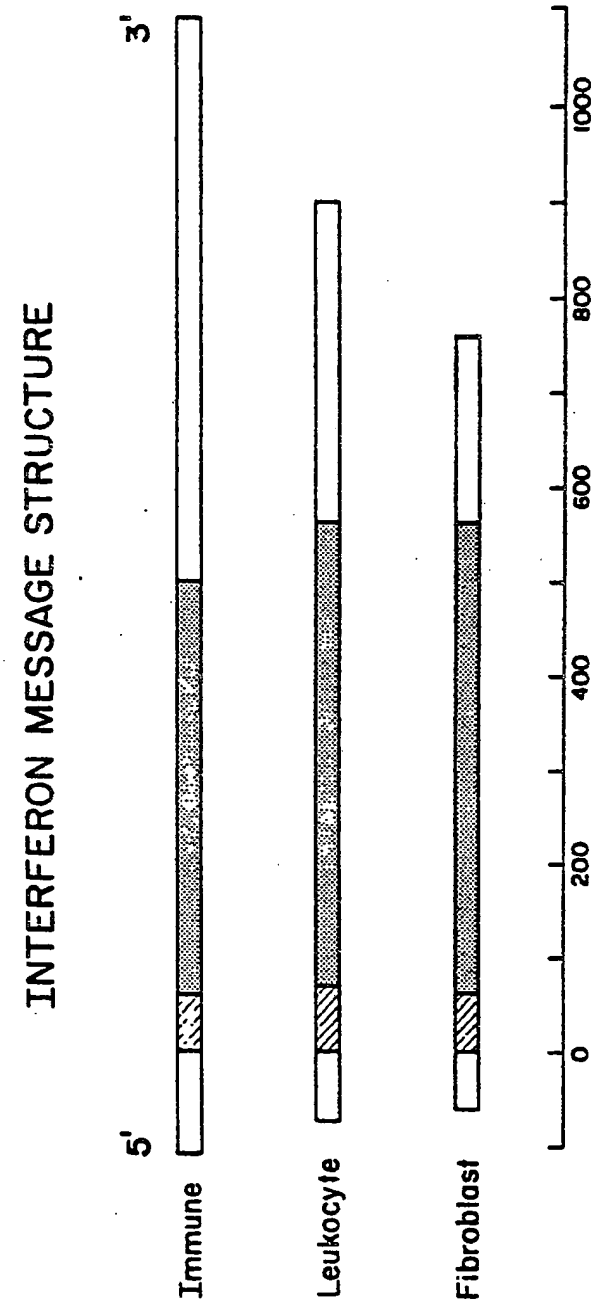

INTERFERON MESSAGE STRUCTURE
5'
3'
Immune
Leukocyte
Fibroblast
0
200
400
600
800
1000

FIG. 6.

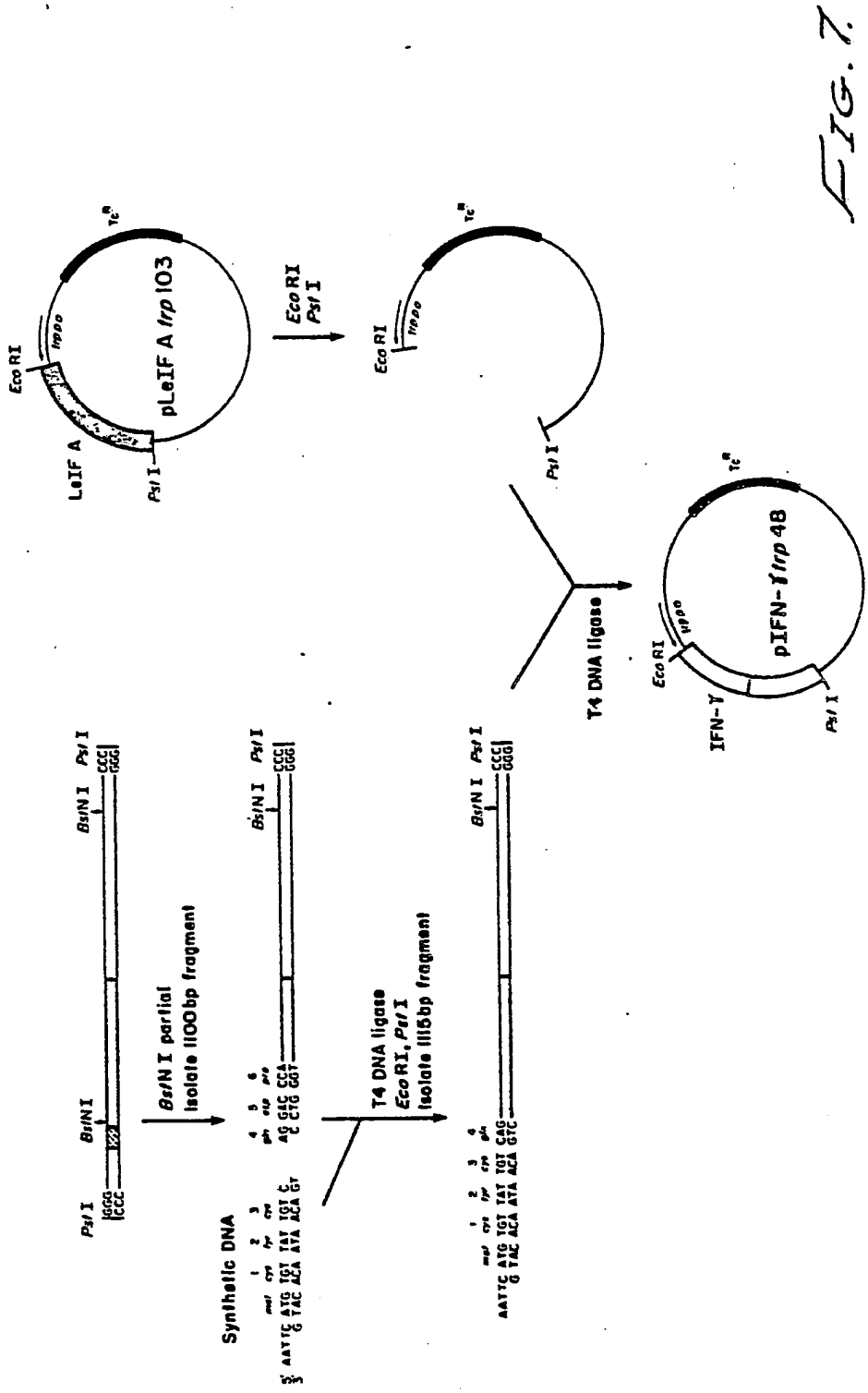

Eco RI
LeIF A
Psi I
pLeIF A trp 103
Eco RI
Psi I
Eco RI
Psi I
T4 DNA ligase
Eco RI
IFN-γ
Psi I
pIFN-γ trp 48
BstN I partial
isolate 1100bp fragment
BstN I
Psi I
Synthetic DNA
T4 DNA ligase
Eco RI, Psi I
isolate 1115bp fragment

FIG. 7.

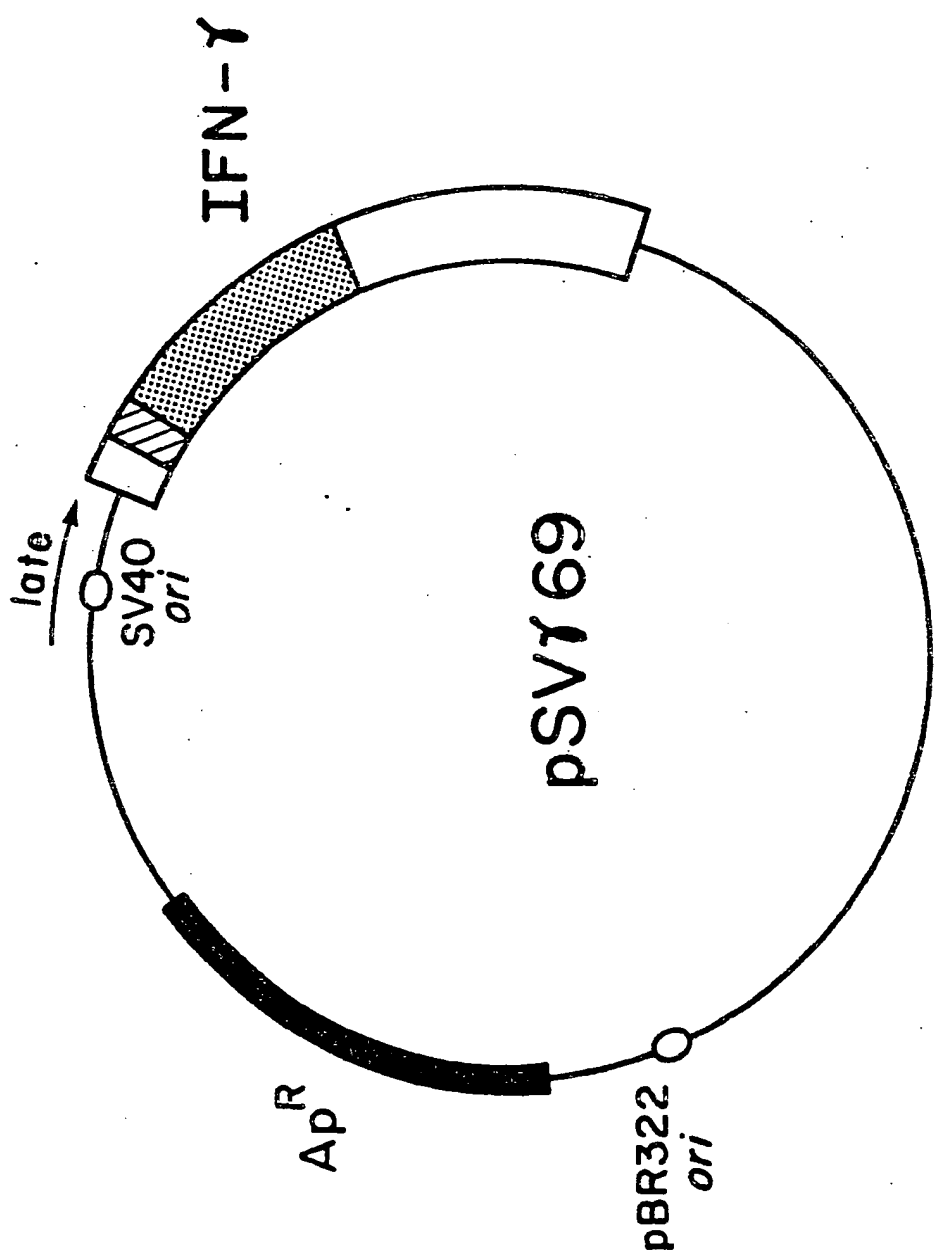



Fig. 8.

# SOUTHERN HYBRIDIZATION

*Eco* RI Digests of Eight Different
Human Genomic DNAs
vs.
Clone 69 Probe

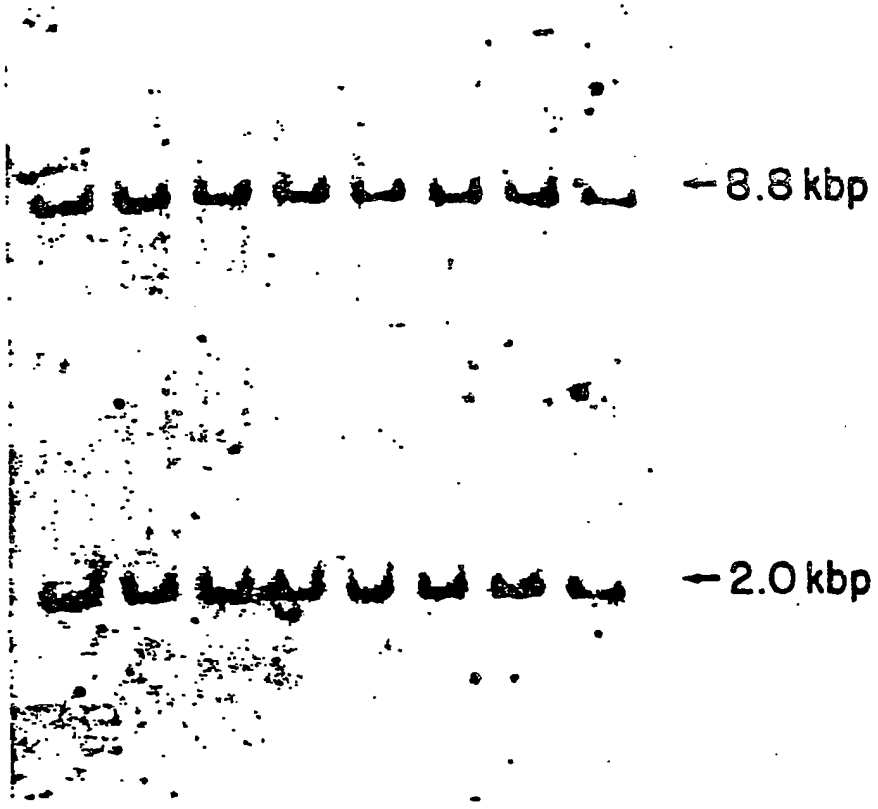



FIG. 9.

# SOUTHERN HYBRIDIZATION

## Digests of Human Genomic DNA

vs.

## Clone 69 Probe

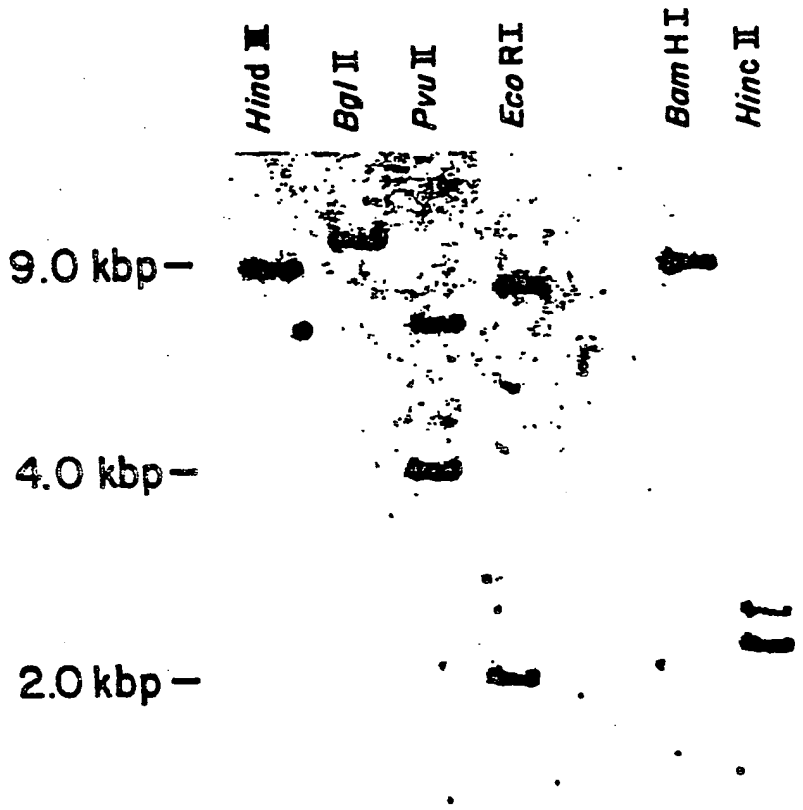



FIG. 10.

# THE INSERTION OF AN EcoRI SITE IN THE 5' FLANKING DNA OF THE 3-PHOSPHOGLYCERATE GENE OF YEAST

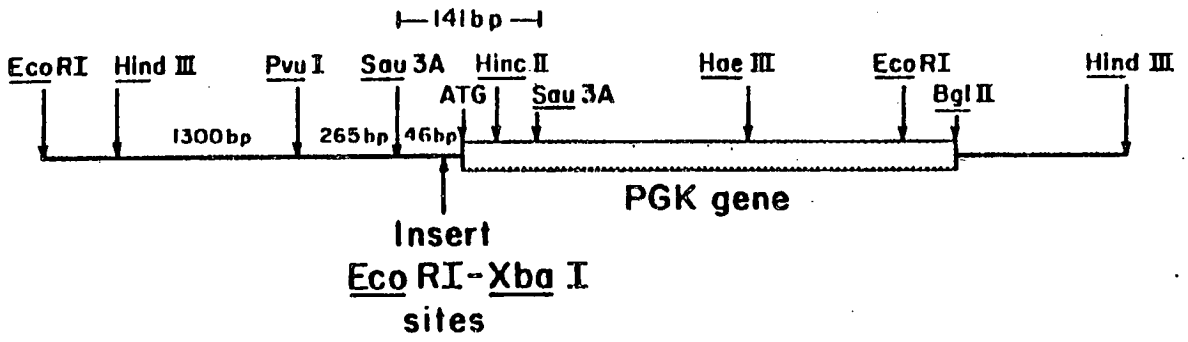



FIG. 11



EP 0 077 670 B1

DNA SEQUENCE OF THE 5' END
OF THE YEAST 3-PHOSPHOGLYCERATE KINASE
STRUCTURAL GENE AND FLANKING DNA

```
                    -40        -30        -20        -10       -1 MET SER LEU SER SER LYS LEU LEU VAL
5'----------GATCATAAGGAAGTAATTATCTACTTTTTACAACAAATATAAAACA ATG TCT TTA TCT TCA AAG TTG CTC GTC
```

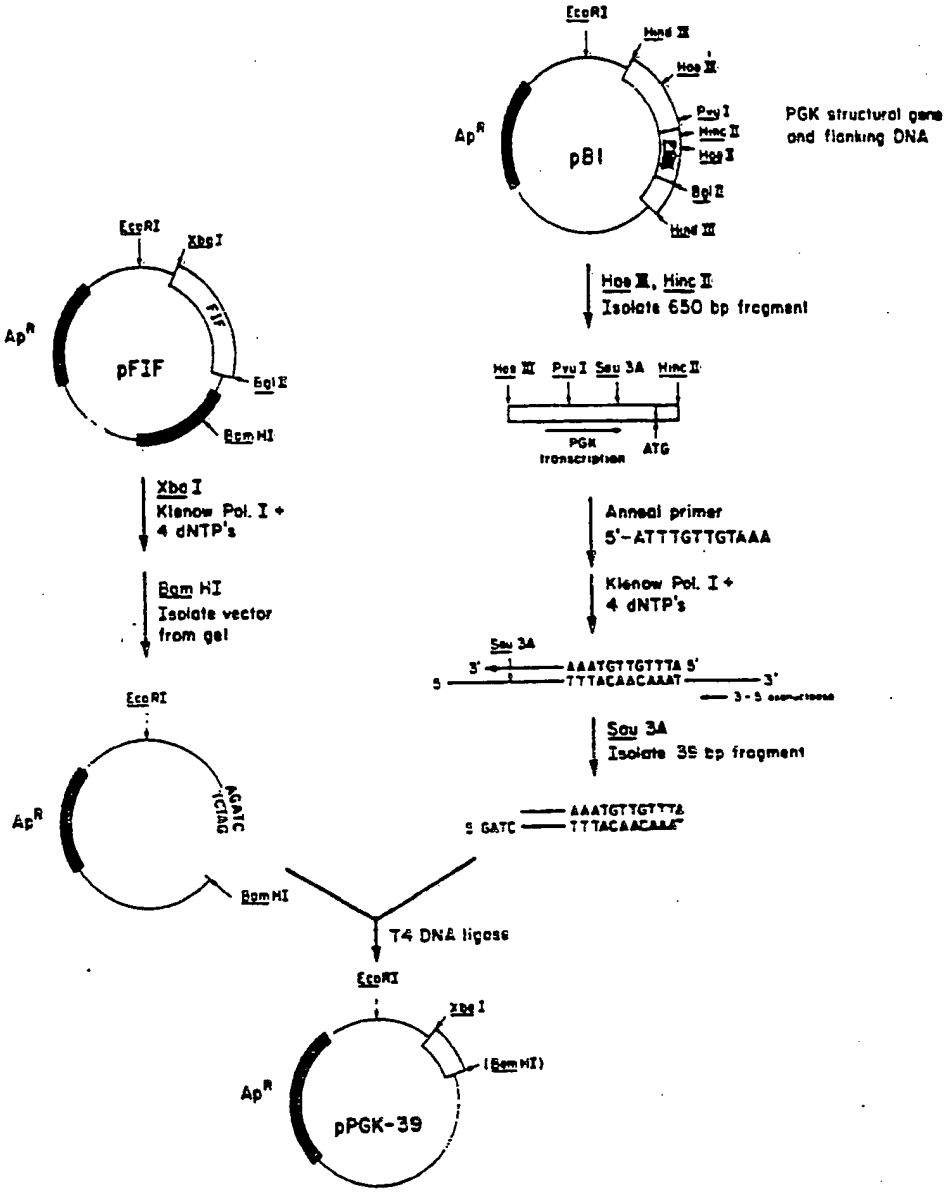

EcoRI
Hind III
Hae III
Pvu I
Hinc II
Hae I
Bgl II
Hind III
Ap^R
pBI
PGK structural gene and flanking DNA
Hae III, Hinc II
Isolate 650 bp fragment
Hae III
Pvu I
Sau 3A
Hinc II
PGK transcription
ATG
EcoRI
Xba I
FIF
Ap^R
pFIF
Bgl II
Bam HI
Xba I
Klenow Pol. I +
4 dNTP's
Bam HI
Isolate vector
from gel
Anneal primer
5'-ATTTGTTGTAAA
Klenow Pol. I +
4 dNTP's
Sau 3A
AAATGTTGTTTA 5'
TTTACAACAAAT
3'
3 - 5 exonuclease
Sau 3A
Isolate 39 bp fragment
AAATGTTGTTTA
GATC
TTTACAACAAAT
EcoRI
AGATC
TCTAG
Ap^R
Bam HI
T4 DNA ligase
EcoRI
Xba I
(Bam HI)
Ap^R
pPGK-39

FIG. 13.

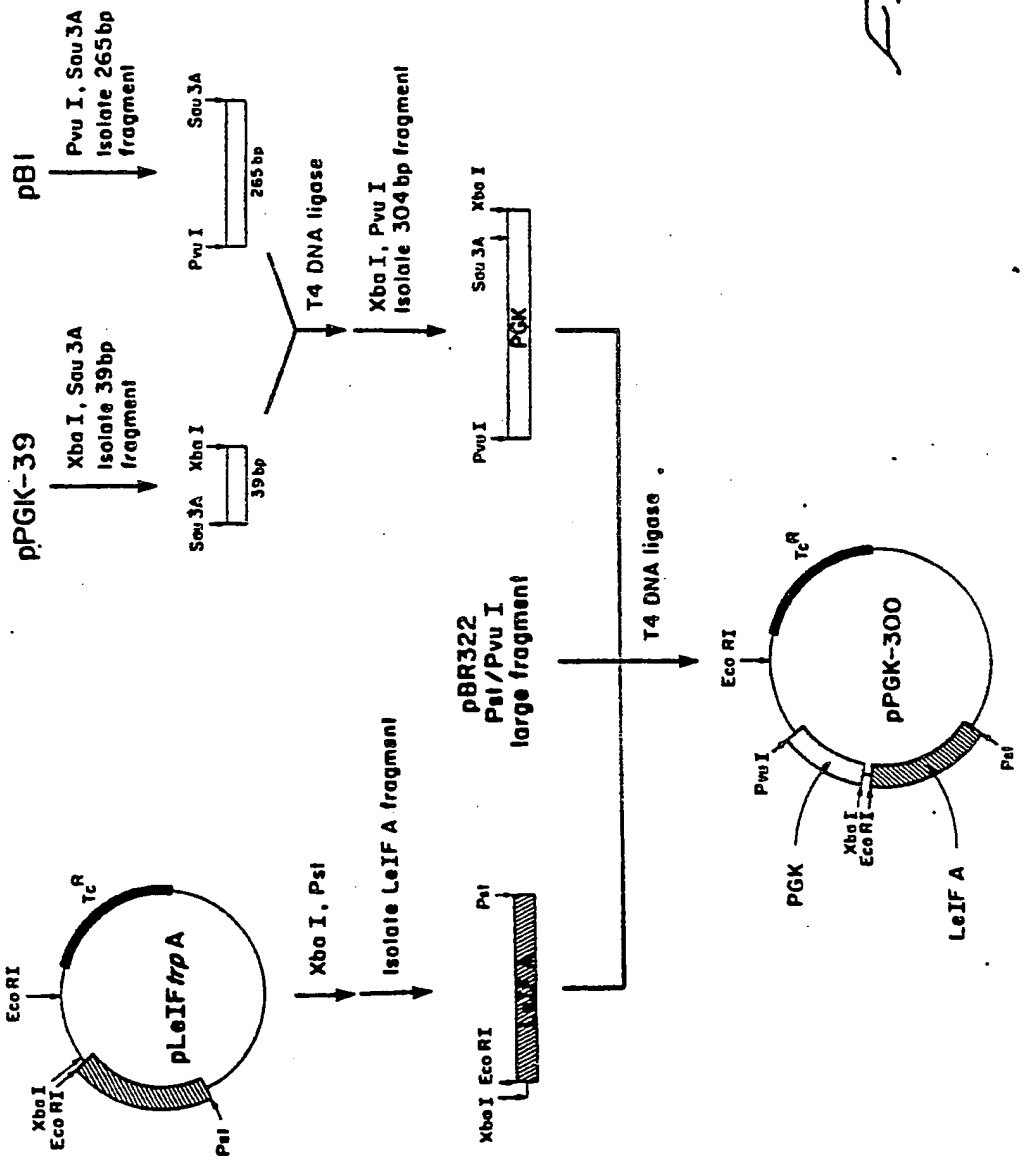

pBI
Pvu I, Sau 3A
Isolate 265bp
fragment
Sau 3A
Pvu I
265 bp
pPGK-39
Xba I, Sau 3A
Isolate 39bp
fragment
Sau 3A
Xba I
39bp
T4 DNA ligase
Xba I, Pvu I
Isolate 304 bp fragment
Sau 3A
Xba I
PGK
Pvu I
pBR322
Pst/Pvu I
large fragment
Xba I
Eco RI
Pst
T4 DNA ligase
Eco RI
Tc^R
pPGK-300
Pvu I
Xba I
Eco RI
Pst
PGK
LeIF A
pLeIF trp A
Eco RI
Tc^R
Xba I
Eco RI
Pst
Xba I, Pst
Isolate LeIF A fragment

FIG. 14.

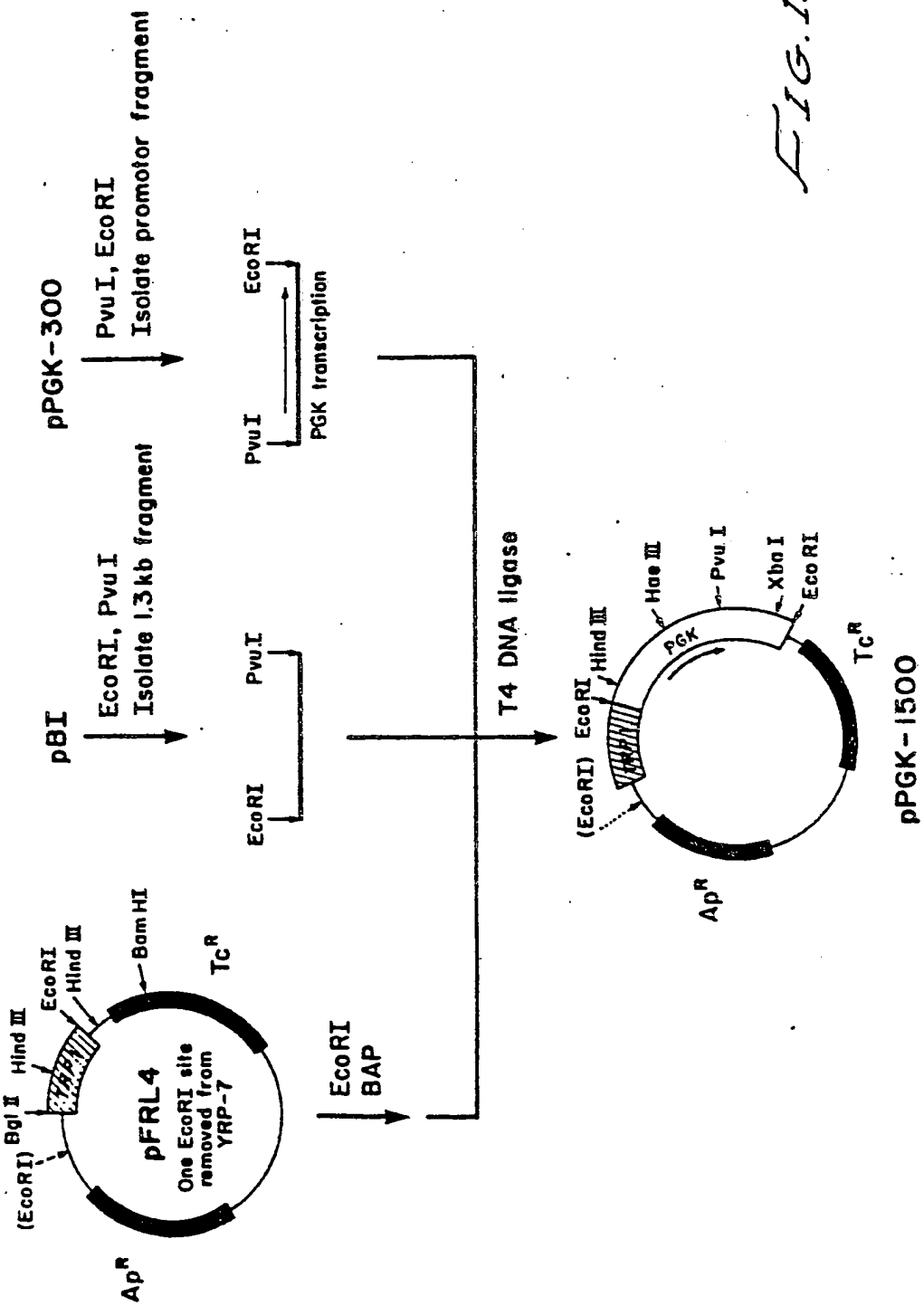

pPGK-300
PvuI, EcoRI
Isolate promotor fragment
EcoRI
PvuI
PGK transcription
pBI
EcoRI, PvuI
Isolate 1.3kb fragment
PvuI
EcoRI
T4 DNA ligase
EcoRI
BAP
pFRL4
One EcoRI site removed from YRP-7
(EcoRI) Bgl II Hind III EcoRI Hind III Bam HI
Ap^R
Tc^R
(EcoRI) EcoRI Hind III Hae III PvuI Xba I EcoRI
PGK
Ap^R
Tc^R
pPGK-1500

FIG. 15.

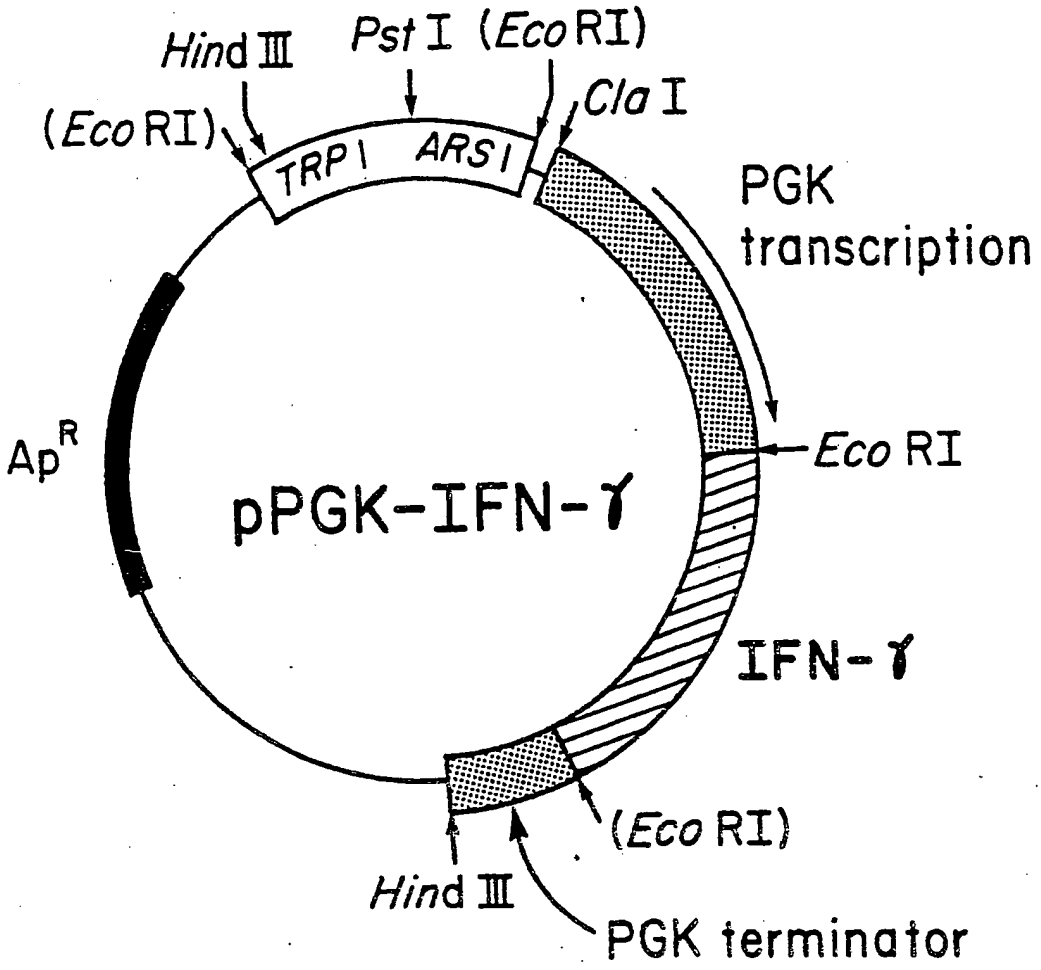

Hind III
Pst I (Eco RI)
(Eco RI)
Cla I
TRP 1
ARS 1
PGK
transcription
Ap^R
pPGK-IFN-γ
Eco RI
IFN-γ
(Eco RI)
Hind III
PGK terminator

FIG. 16.

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